RESPONSES AND MECHANISMS OF A MEDITERRANEAN GRASSLAND ECOSYSTEM TO NUTRIENT ADDITION

CARLA DE ALMEIDA NOGUEIRA

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TESE ELABORADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA FLORESTAL E DOS RECURSOS NATURAIS

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À Rita, ao Vasco e ao Tiago

Tese de doutoramento

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Título: Responses and mechanisms of a Mediterranean grassland ecosystem to nutrient addition.

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Responses and mechanisms of a Mediterranean grassland ecosystem to nutrient addition

PhD in Forestry Engineering and Natural Resources

Abstract

Global changes, resulting from anthropogenic activities, are increasing precipitation variability, drought and nutrient inputs into ecosystems. These global change drivers are expected to induce changes in grassland species richness and composition and functional structure and diversity which may in turn affect ecosystem functioning. This is particularly important for the Mediterranean basin, a climate change hotspot. Understanding how these changes affect grassland structure and functioning is critical to anticipate impacts of global change, improve management actions and develop land management strategies and restoration tools to mitigate grassland degradation.

Through a pot greenhouse experiment, we applied three levels of extended autumn drought and two levels of nitrogen deposition to grassland communities. The severe drought originated a shorter growing season, and led to lower net ecosystem exchange and gross primary productivity, which translated into reduced productivity. Drought induced changes in functional group proportion and delayed plant phenology. Nitrogen addition did not affect productivity, diversity or phenology. However, nitrogen interacted with the severe drought treatment to attenuate the negative effects on total carbon fluxes.

A 5-year nutrient addition field experiment was also conducted. Nitrogen, phosphorus and potassium were added in a factorial way to establish three treatments of one, two and three added nutrients, including controls. Grassland productivity was co-limited by multiple nutrients and precipitation. Nutrient addition decreased species richness and interacted with climatic variability to alter functional group composition. Resilience to disturbance was not affected by nutrient addition, as resistance that decreased with nutrient enrichment due to lower species richness was cancelled out by increased recovery due to the dominance of competitive graminoids. Community functional structure was affected by nutrient addition and precipitation and, for some traits, by their interaction and was the main

determinant of productivity. Functional diversity was affected by precipitation, with drought negatively affecting community functional diversity.

Keywords: climatic variability; functional traits; Mediterranean grassland; nutrients; productivity.

Respostas e mecanismos de um ecossistema de pastagem Mediterrânico à adição de nutrientes

Doutoramento em Engenharia Florestal e dos Recursos Naturais

Resumo

As actividades antropogénicas estão a aumentar a variabilidade da precipitação, a incidência da seca e disponibilidade de nutrientes. Estes factores poderão originar alterações na riqueza e composição em espécies das pastagens, bem como na sua estrutura e diversidade funcional, o que poderá afectar o seu funcionamento. Compreender como estas mudanças afectam a estrutura e o funcionamento das pastagens da bacia Mediterrânica, um *hotspot* para as alterações climáticas, é fundamental para antecipar os impactos e desenvolver estratégias de gestão e ferramentas de restauro para mitigar a sua degradação.

Num ensaio em vaso, aplicamos três níveis de seca de outono e dois níveis de deposição de azoto numa pastagem. A seca severa levou a uma redução da época de crescimento originando uma redução nas trocas gasosas líquidas de CO_2 e água do ecossistema e na produtividade primária líquida, traduzindo-se numa redução da produtividade. A seca induziu alterações na proporção dos grupos funcionais e atrasos na fenologia. A adição de azoto interagiu com a seca severa para atenuar os efeitos negativos desta nos fluxos totais de CO_2.

Durante cinco anos foi conduzido um ensaio de campo. Azoto, fósforo e potássio foram adicionados de forma factorial, estabelecendo-se três tratamentos de um, dois e três nutrientes adicionados, incluindo controlos. A produtividade da pastagem mostrou ser co-limitada por múltiplos nutrientes e precipitação. A adição de nutrientes levou ao decréscimo da riqueza e interagiu com a variabilidade climática para alterar a proporção dos grupos funcionais. A resiliência à perturbação não foi afectada pela adição de nutrientes devido a uma resposta antagónica da resistência e da recuperação. Embora a resistência tenha decrescido com a adição de nutrientes, devido a uma menor riqueza, a dominância de gramíneas competitivas terá favorecido a recuperação das comunidades.

A estrutura funcional foi afectada pela adição de nutrientes e precipitação e para alguns atributos funcionais pela sua interacção, determinando a produtividade das comunidades. A diversidade funcional foi afectada pela precipitação, tendo a seca um efeito negativo na diversidade funcional.

Palavras-chave: atributos funcionais; nutrientes; pastagem Mediterrânica; produtividade variabilidade climática.

Respostas e mecanismos de um ecossistema de pastagem Mediterrânico à adição de nutrientes

Resumo alargado

Os cenários de alterações climáticas prevêem um aumento na frequência e intensidade das secas. O aumento do uso de fertilizantes, especialmente azoto (N) e fósforo, bem como a deposição de N proveniente da queima de combustíveis fósseis estão a aumentar a disponibilidade destes nutrientes nos ecossistemas. Estas alterações poderão afectar a composição específica e diversidade das pastagens, bem como a sua estrutura e diversidade funcional, o que poderá comprometer o seu funcionamento. É crucial compreender o impacto destas alterações globais nas pastagens em particular, na bacia Mediterrânica, que é um *hotspot* para as alterações climáticas. É decisivo compreender estas respostas para antecipar os impactos e desenvolver estratégias de gestão e ferramentas de restauro que possam mitigar a sua degradação. Para tal foram estabelecidos dois ensaios, um em estufa, durante um ano (Capítulos 2 e 3), e outro em campo com a duração de cinco anos (Capítulos 4 e 5; Apêndice).

No ensaio em estufa, foram utilizados 48 vasos onde foi colocado solo com banco de sementes de pastagem e aplicados os seguintes tratamentos: três níveis de seca de outono (sem seca, moderada e severa) e dois níveis de deposição de N (sem adição de N e adição de N a uma taxa de 12 kg ha ano^{-1}). Foram consideradas oito réplicas por tratamento (Capítulos 2 e 3). O banco de sementes foi recolhido numa pastagem semi-natural anual, na Companhia das Lezírias (CL). A quantidade de N adicionada representa a taxa média de deposição atmosférica de N projectada para 2050, para a bacia Mediterrânica. Avaliou-se a produtividade, a proporção dos grupos funcionais (GF; em gramíneas, leguminosas e outras espécies), a diversidade, a fenologia e os fluxos de CO_2 e água das comunidades.

O prolongamento da seca de outono afectou as respostas da comunidade. A seca severa diminuiu a produtividade, reflexo de uma menor duração da estação de crescimento e menor acumulação de graus dias de crescimento. Embora em Abril, a produtividade dos

controlos fosse substancialmente maior do que dos tratamentos de seca, em Maio as comunidades sujeitas a seca moderada tiveram um acréscimo de 17% na sua produtividade, recuperando assim para níveis semelhantes ao dos controlos. As comunidades sujeitas a seca severa registaram um aumento de 42% produtividade. No entanto a sua produtividade foi menor do que a dos controlos.

O tratamento de não seca favoreceu o estabelecimento do GF “outras espécies” (p.ex. espécies com roseta), enquanto as gramíneas foram favorecidas por uma seca moderada (Capítulo 2). Contrariamente ao esperado, a seca de outono não teve um efeito negativo na riqueza em espécies. A grande abundância de espécies com roseta nos controlos e a sua vantagem competitiva no estabelecimento após a rega poderá explicar a redução na riqueza em espécies.

A seca originou uma menor acumulação de graus dias de crescimento, o que determinou uma menor proporção de indivíduos com flores e frutos ou sementes (Capítulo 2). As diferenças na fenologia foram particularmente negativas para o GF “outras espécies”, dominante na nossa comunidade. Desta forma, em anos futuros e em condições prevalecentes de seca de outono, a composição florística poderá ser alterada e dominada por gramíneas, cuja proporção de indivíduos com flores não foi tão afectada pela seca e eventualmente vir a comprometer o funcionamento das comunidades de pastagem.

A adição de N não teve qualquer efeito na produtividade, grupos funcionais e fenologia (Capítulo 2) provavelmente devido a uma co-limitação da produtividade da pastagem por outros nutrientes, o que está de acordo com os resultados obtidos no ensaio em campo, embora as quantidades adicionadas tenham sido substancialmente mais elevadas (Capítulo 4).

A seca de outono teve um efeito negativo nos fluxos de CO_2 e água das comunidades (Capítulo 3). A maior duração da época de crescimento nas comunidades controlo explica as diferenças na produtividade primária líquida (PPL) e a maior PPL total, reflectindo-se na produtividade. A eficiência no uso da água das comunidades foi negativamente afectada pela seca severa (Capítulo 3). Estas comunidades mais jovens germinaram mais tardiamente, e tendo possivelmente uma capacidade fotossintética mais elevada, desenvolveram-se num contexto em que a necessidade em água seria maior (i.e. temperaturas mais elevadas). A diminuição na eficiência do uso da água e na PPL com o

prolongamento da seca de outono poderá comprometer a resiliência destas comunidades de pastagem.

A soma dos fluxos de CO_2 e de água ao longo da estação de crescimento diminuíram com a seca de outono (Capítulo 3). No entanto, a adição de N atenuou o efeito negativo da seca severa nos fluxos de CO_2. Possivelmente uma menor taxa de mineralização do N no início deste tratamento devido as temperaturas mais baixas, bem como a presença de uma comunidade com maiores taxas de crescimento no inicio da primavera poderão explicar a razão pela qual o N teve um efeito significativo nestas comunidades.

Em Junho de 2012 foi instalado um ensaio de nutrição numa zona de pastagem semi-natural na CL. O ensaio foi estabelecido de acordo com o protocolo do *Nutrient Network* (www.NutNet.org). Numa área vedada, foram instalados três blocos, num total de 36 parcelas, cada com 5 m x 5 m. N, P e potássio foram adicionados de forma factorial, estabelecendo-se três tratamentos de um, dois e três nutrientes adicionados, incluindo controlos. Os três nutrientes foram adicionados, anualmente no início da época de crescimento, a uma taxa elevada de 100 kg N ha yr^{-1}. Anualmente, de 2013 a 2017, a riqueza em espécies, composição florística e produtividade foram estimadas. Foi também determinada a estrutura e a diversidade funcional da comunidade. O primeiro ano (2013) foi um ano com precipitação normal, o segundo (2014) foi um ano mais húmido, com uma precipitação total 15% acima da média para a região para o período de 1961 a 1990. Os três anos seguintes 2015, 2016 e 2017 foram anos secos, com precipitação total 34%, 16% e 32% abaixo da média.

Verificou-se que a produtividade da comunidade foi co-limitada pela precipitação e múltiplos nutrientes (Capítulo 4). A adição de três nutrientes levou consistentemente (excepto em 2015) a um aumento da produtividade, e anos mais secos a uma menor produtividade. A adição de três nutrientes favoreceu, em geral, o desenvolvimento de espécies competidoras por recursos, como as gramíneas. De facto estas espécies apresentaram, em média, uma área foliar específica e altura máxima maiores comparativamente a outras espécies presentes na comunidade (Capítulo 5). No entanto, a interacção da adição de nutrientes e do ano climatológico determinou a proporção de gramíneas e do GF “outras espécies”. Por exemplo, invernos mais húmidos terão facilitado o estabelecimento do GF “outras espécies”, enquanto as gramíneas terão tido uma vantagem competitiva em anos mais secos. A sua maior área foliar específica terá

estado associada a um melhor desempenho em anos de seca. A proporção de leguminosas não foi afectada pelo número de nutrientes, no entanto a presença de N nos tratamentos, bem como a seca tiveram um efeito negativo naquele grupo.

A adição de três nutrientes levou, consistentemente, a uma redução da riqueza (Capítulo 4). No entanto, foi num ano de seca que se registou a maior redução no número de espécies, em todos os tratamentos, sugerindo que as secas são determinantes na riqueza das pastagens Mediterrânicas independentemente da sua disponibilidade em nutrientes. Um ano de seca também teve um efeito negativo na diversidade funcional da maior parte dos atributos funcionais das plantas (AFP) (Capítulo 5). Embora a estrutura funcional tenha sido afectada pela adição de nutrientes e pela precipitação e para alguns AFPs pela sua interacção, a diversidade funcional da comunidade foi essencialmente determinada pela precipitação.

No início da época de crescimento do terceiro ano do ensaio (2015), houve uma entrada imprevista de gado bovino nas parcelas. O pastoreio intenso e a seca possibilitaram assim, o estudo do efeito da adição de nutrientes na resistência, recuperação e resiliência da pastagem à perturbação (Capítulo 4). Apesar da sua menor riqueza, a dominância de gramíneas, eficientes no uso de recursos, terá contribuído para a estabilidade da produtividade ao longo do estudo. Verificou-se que a adição de nutrientes não afectou a resiliência da produtividade da pastagem devido a uma resposta antagónica da resistência e da recuperação. Se por um lado a menor riqueza em espécies, no tratamento com três nutrientes, e um maior desenvolvimento da vegetação, com provável presença de plantas maiores e mais facilmente acessíveis ao gado e com maior palatabilidade, em comparação com os outros tratamentos terá induzido numa menor resistência à perturbação. Por outro lado, a dominância, de gramíneas no tratamento com três nutrientes teve um efeito positivo na recuperação.

Os AFPs permitem de forma mecanística associar as espécies ao funcionamento do ecossistema. De facto, a estrutura funcional das comunidades foi o principal determinante da produtividade (Capítulo 5). No entanto, embora os atributos mais abundantes tenham tido um efeito significativo na produtividade (p.ex. gramíneas, espécies com roseta), verificou-se também que atributos menos abundantes podem ser determinantes na produtividade (p.ex. espécies prostradas).

A variabilidade na estrutura e diversidade funcional observada ao longo de anos climatológicos contrastantes bem como ao longo do gradiente de disponibilidade de nutrientes, mostra claramente as soluções alternativas desenvolvidas pelas espécies que compõem as pastagens Mediterrânicas (Capítulo 5). É esta variabilidade que contribui não apenas para manter seu funcionamento, como também a sua resiliência. No entanto, se como previsto, os episódios de seca forem mais frequentes, é previsível uma fragilização da resiliência destas pastagens com base na redução observada na sua diversidade funcional num ano de seca.

Esta tese contribuiu para uma melhor compreensão do papel fundamental da precipitação de outono na composição, produtividade e fenologia, bem como nos fluxos de CO_2 e água das pastagens Mediterrânicas. Contribuiu para compreender as respostas destas comunidades a um aumento de disponibilidade de nutrientes e variabilidade climática, chamando a atenção para a co-limitação por múltiplos nutrientes. Este trabalho revelou ainda alterações importantes na estrutura funcional das pastagens devido à adição de nutrientes e precipitação, mostrando a importância da precipitação e da seca na diversidade funcional das pastagens Mediterrânicas.

Contents

CHAPTER 1

General Introduction

1 General Introduction

Grasslands may be defined as “land covered with herbaceous plants with less than 10% tree and shrub cover” (UNESCO, 1973) or with a broader scope, as encompassing not only non-woody grasslands but also savannas, woodlands, shrublands, and tundra (White et al., 2000). Grasslands are among the largest ecosystems in the world. They occupy approximately 40% of the earth’s terrestrial area excluding Greenland and Antarctica, and are found most commonly in semi-arid zones (28% of the world’s grasslands), followed by humid (23%), cold (20 %), and arid zones (19%) (White et al., 2000).

Grasslands provide a wide range of ecosystem services such as forage for wild and domestic animals, carbon sequestration, soil protection, water and climate regulation and beyond the preservation of plant species diversity, grasslands also support a rich fauna (White et al., 2000; Faber-Langendoen and Josse, 2010). However, at a global scale, Mediterranean ecosystems, including grasslands, are among the most sensitive to global changes such as increased drought occurrence and temperature and nitrogen deposition and are simultaneously expected to experience the greatest biodiversity loss (Sala et al., 2000). Mediterranean grasslands, in particular, feature high species diversity (Peco et al., 1998; Díaz-Villa et al., 2003) which is essential to support the provision of ecosystem services such as productivity or soil nitrogen cycling (Hector et al., 1999; Caldeira et al., 2005; Hector and Bagchi, 2007).

The high intra- and inter-annual variability in precipitation, along with prolonged summer drought which characterizes Mediterranean climate regions, drives the patterns of grassland diversity and productivity (Murphy, 1970; Pitt and Heady 1978; Peco et al., 1998; Eviner, 2006). Mediterranean grasslands consist mainly of annual C3 species (Ehleringer et al., 1997) that spend the dry hot summer as seeds to avoid drought (Pitt and Heady, 1978; Peco et al., 1998; Chaves et al., 2002). In these grasslands, the growing season starts with the first significant rains in autumn, continues until spring when longer day length, mild temperatures and precipitation accelerate vegetation growth (e.g. Xu and Baldocchi, 2004; Eviner, 2016). In late spring/early summer, when temperatures are high and there is no precipitation, they senesce (Bartolome, 1979; Xu and Baldocchi, 2004). The timing, distribution and amount of precipitation during the grassland growing season

relates better to productivity than total annual precipitation (Eviner, 2016). The dominance of annuals species renders these grasslands high sensitivity to fluctuations in abiotic (e.g. climate, nutrient availability) and biotic (e.g. herbivory) factors which stresses the need for an understanding of grassland functioning in the context of environmental changes (e.g. climate change and increased nutrient availability), in order to develop strategic conservation and land management policies.

Climate change and nutrient inputs are two main drivers of global change (e.g. Sala et al., 2000; Knapp et al., 2002; Peñuelas et al., 2013; Dornelas et al., 2014). Anthropogenic climate change is relevant world-wide and supported by a broad number of studies and observations (e.g. Trenberth et al., 2014; IPCC, 2014; Christidis et al., 2015). Climate models projections show significant agreement, in forecasting increased temperatures all over Europe, with strongest warming and more intense and longer meteorological droughts in Southern Europe (Kovats et al., 2014) (Fig. 1). Among twenty-six regions across the globe, the Mediterranean region emerges as a primary climate change hotspot, where by the latter decades of the twenty-first century this region is projected to experience the greatest drying (Giorgi, 2006). Indeed, over the period 1902-2010, and between November and April, the Mediterranean basin region has already experienced an accentuated water deficit, with increased drought frequency since 1970 (Hoerling et al., 2012). Since the early 1960s, precipitation in March (spring) has been continuously declining in Portugal (Paredes et al., 2006) and more recently (2011-2012) the Iberian Peninsula has experienced one of the most severe winter droughts (Trigo et al., 2013). In particular, climate change scenarios for Portugal reveal a decrease in mean precipitation in autumn and spring, extending the duration of the dry season (Miranda et al., 2002; Costa et al., 2012). Predictions, also point out to increased precipitation variability, and an increase in maximum and minimum temperatures (Miranda et al., 2002; Ramos et al., 2011; Costa et al., 2012).

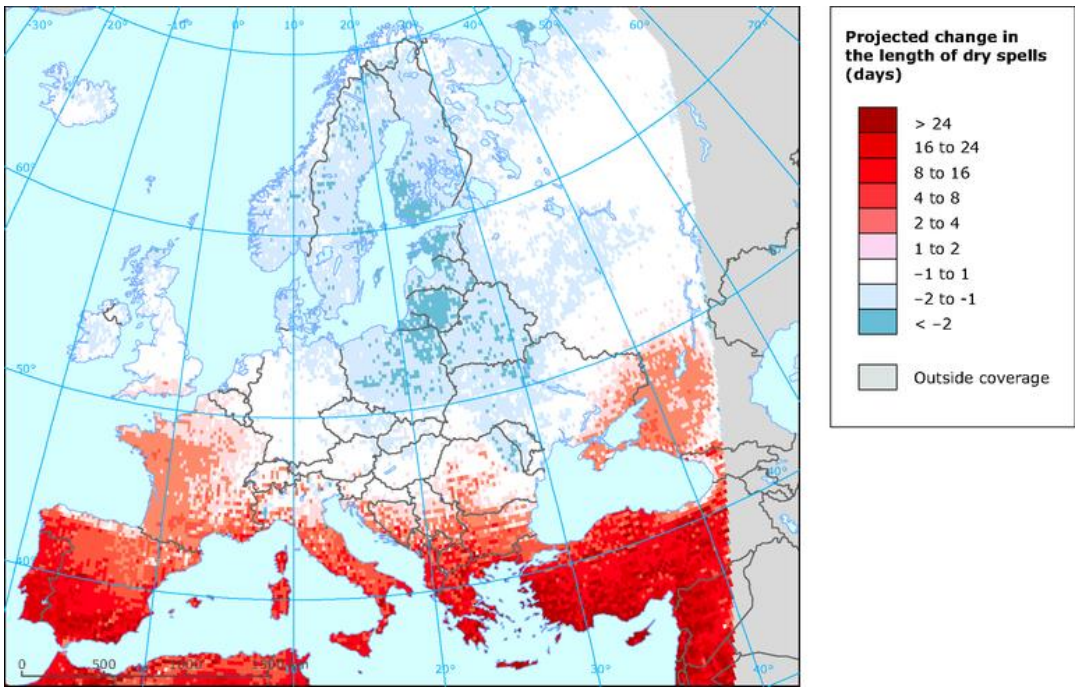

Fig. 1 Projected changes in the length of dry spell (in days) from 1971-2000 to 2071–2100 for the RCP8.5 scenario based on the ensemble mean of different regional climate models (RCMs) nested in different general circulation models (GCMs). Source: (http://www.eea.europa.eu/legal/copyright).

From 1860 to the early 1990s, anthropogenic reactive nitrogen (N) (*sensu* Galloway et al. 2004) increased globally from approximately 15 to 156 Tg N yr^{1} due to the Haber-Bosch process (fertilizer production), cultivation of N-fixing species and rice and fossil fuel combustion. The projected values for 2050 are of ca. 267 Tg N yr^{-1} (Galloway et al., 2004). Also, since the industrial revolution, phosphorus (P) inputs have increased from <0.3 to 16 Tg P yr^{-1}, mainly due to the application of mineral fertilizers and the use of livestock slurry and manure (Peñuelas et al., 2012; 2013) (Fig. 2). To a lesser extent, P input in natural ecosystems can result from excess P fertilizer being redistributed into adjacent ecosystems via runoff or transported via freshwater bodies, and by atmospheric deposition of mineral aerosols of dust from P fertilizers (Peñuelas et al., 2013). Thus the consequences of increased nutrient flows in the biosphere have become one of the major components of global change (e.g. Sala et al., 2000; Elser et al., 2007; Harpole et al., 2011). Nonetheless, it remains poorly understood how multiple nutrient interactions regulate primary productivity and impacts species diversity in natural systems, including grasslands (e.g. Harpole et al., 2011, 2016; Fay et al., 2015; Flores-Moreno et al., 2016).

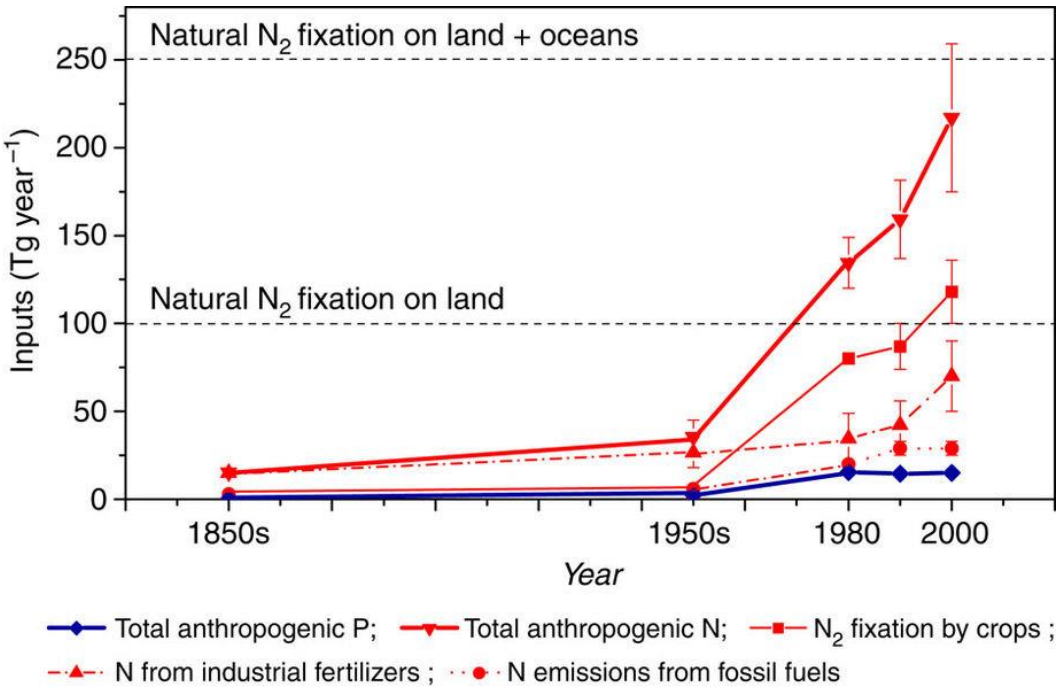

Fig. 2 Anthropogenic reactive N and P inputs to the biosphere (mean ± s.e.; Tg per year) since the industrial revolution (1860). Source: Peñuelas et al. (2013).

1.1 Grassland productivity response to precipitation

Across broad spatial scales and when temperature is not limiting, precipitation is a key driver of aboveground net primary productivity (ANPP) (Churkina and Running, 1998; Knapp and Smith, 2001; Huxman et al., 2004a). Comparing different biomes (desert, artic/alpine, grassland and forest) across North America, Knapp and Smith, (2001) observed that grasslands displayed the highest variability in ANPP due to inter-annual precipitation variability. Mediterranean grasslands, in particular, are among the most responsive to changes in precipitation (Huxman et al., 2004a; Cherwin and Knapp, 2012; Knapp et al., 2015). The high inter-annual precipitation variability characteristic of Mediterranean climates coupled with the dominance of plants species with an annual life cycle, makes ANPP of Mediterranean grasslands extremely variable (Eviner, 2016). For instance, in a semi-natural grassland in Évora, Portugal, along four years, ANPP ranged from 0.14 kg m^{-2} during the drier year to 0.80 kg m^{-2} during the wetter year (Jongen et al., 2011). Therefore, predicted changes in precipitation regimes (Miranda et al., 2002; Lehner et al. 2006; Costa et al., 2012) are expected to have important consequences on Mediterranean grassland productivity (e.g. Zavaleta et al., 2003; Miranda et al., 2009a,b; Jongen et al., 2011; Kreyling et al., 2017).

1.2 Grassland composition and diversity response to precipitation

Along with changes in productivity, variations in precipitation may also induce changes in diversity, composition and structure of plant communities (Pitt and Heady, 1978; Espigares and Peco, 1993, 1995; Pugnaire and Lázaro, 2000; Prieto et al., 2009; Miranda et al., 2009a, b; Carmona et al., 2015; Rota et al., 2017) with expected stronger effects where water availability is already limiting (Knapp et al. 2002).

The timing and temperature at which first autumn rains occur, as well as quantitative changes in soil water availability influence germination and seedling establishment, with concomitant effects on species composition and diversity of communities, due to species differential germination requirements (Pitt and Heady, 1978; Espigares and Peco, 1993, 1995; Miranda et al., 2009a; Eviner, 2016). Nonetheless, studies on Mediterranean grassland ecosystems (e.g. Zavaleta et al., 2003; Dukes et al., 2005; Gerten et al., 2008; Jongen et al., 2013) have mainly focused on the effect of increasing precipitation variability and less on the effects of a delayed onset of autumn rainfalls on productivity and diversity (but see Miranda et al., 2009a). Drought periods after first autumn rains (i.e. after germination) may also affect floristic composition due to a differential effect on seedling mortality which, also may contribute to decrease richness (Espigares and Peco, 1995). Together with herbivory and pathogen attack, drought has been identified as one of the major causes of seedling mortality (Moles and Westoby, 2004), which may decrease species richness and hamper grassland diversity recovery (Tilman and El Haddi, 1992). On the other hand, spring precipitation may strongly impact and alter seed production (Ewing and Menke, 1983) which may influence grassland species composition and diversity the following year (Dudney et al., 2017). A more frequent occurrence of long droughts, as predicted by climate models for the Mediterranean region, may thus decrease seed quantity and diversity, contributing to plant diversity loss as suggested by Zeiter et al. (2016) for a semi-natural grassland, in southern Switzerland.

1.3 Grassland productivity and diversity response to N enrichment

Atmospheric deposition of reactive N increases the availability of a major limiting nutrient for plant production (Vitousek and Howarth 1991; Bobbink et al., 1998). The consequent

increased plant growth may enhance competition for light (Hautier et al., 2009), favoring taller species at maturity and decreasing species richness (Tilman, 1987). However, on very nutrient poor soils, species number may increase because of invasion of species that were not tolerant to the original nutrient poor soils (Bobbink et al., 1998). In the long term, N deposition may accelerate the leaching loss of base cations, lowering soil pH and increasing the concentration of toxic metals (Bobbink et al., 1998; 2010; Stevens et al., 2010b). This situation may further reduce the number of species (Bobbink et al., 1998; Stevens et al., 2010a, b). As species richness is linked to an array of ecosystem functions (Isbell et al., 2011;Tilman et al., 2014), a decrease in species richness with N deposition may have important consequences for ecosystem productivity (e.g. Hector et al., 1999; Isbell et al., 2013; Duffy et al., 2017), stability or resilience (e.g. Caldeira et al., 2005; Tilman et al., 2014; Volaire et al., 2014), resistance to drought (e.g. Tilman and Downing, 1994) and ecosystem services, such as nutrient cycling (e.g. Hector and Bagchi, 2007).

Apart from a decrease in species richness, community composition and structure may also respond to N addition (e.g. Zavaleta et al., 2003; Suding et al., 2005; Xia and Wan, 2008), from which often emerges new dominance patterns (Collins et al., 2008). For instance, N enrichment may decrease forb and enhance grass abundance (Foster and Gross, 1998; Zavaleta et al., 2003; Xia and Wan, 2008; DeMalach et al., 2017a), as it may also increase the risk of some groups to be lost (e.g., N-fixing and rare species) (Suding et al., 2005). Although it is possible to identify consistent patterns in species response to N enrichment (Xia and Wan, 2008; DeMalach et al., 2017a), they often depend on the abiotic or biotic context (Pennings et al., 2005). For instance, Clark et al. (2007) showed that in a review of 23 N enrichment experiments, representing major herbaceous ecosystems in temperate North America, greater species loss occurred in communities with lower cation exchange capacity, colder regional temperatures, and stronger production responsiveness to N. In fact, the magnitude of productivity and diversity responses can be community-specific (Gough et al., 2000).

The environmental context, in particular the amount and distribution of precipitation, significantly determines grassland responses to N addition (e.g. Dukes et al., 2005; Harpole et al., 2007; Xia and Wan, 2008; Simkin et al., 2016; DeMalach et al., 2017a). In Mediterranean regions, water availability is the main factor limiting net primary productivity (Churkina and Running, 1998) and influences, the diffusion of soil nutrient to plant roots

and for instance N mineralization (Jarvis et al., 2007; Eviner and Firestone, 2007; Cleland and Harpole, 2010)

Future climate change scenarios project for the Mediterranean region more severe droughts interspersed with increased occurrence of extreme precipitation events (Miranda et al., 2002; Kovats et al., 2014). These forecasted changes will have a strong influence on the dynamics of N accumulation and N fluxes, which will affect the level of N deposition at which changes in the structure and function of ecosystems occur (i.e. critical load values) (Fenn et al., 2010). Indeed, extended dry periods may lead to higher N accumulation in soil and plant surfaces and so to potentially more negative effects (such as toxicity) as soon as water becomes available (Ochoa-Hueso et al., 2011) or to positive effects if N content in the soil is low (Bobbink et al., 1998).

Although N deposition has been associated with declines in species richness especially in temperate Europe (e.g. Stevens et al., 2010a, b) and North America (e.g. Isbell et al., 2013; Simkin et al., 2016), an understanding of plant communities response to N deposition in the Mediterranean basin has been less investigated (Bobbink et al., 2010; Ochoa-Hueso et al., 2011). This is of relevant concern, as this region is recognized as a biodiversity hotspot (Myers et al., 2000), and the percentage area of biodiverse sites, receiving between 10 and 15 Kg N ha^{-1} yr^{-1} (which are in the range of critical loads for many of these ecosystems) is projected to increase up to 70% by 2050 compared to 13% in the mid- 1990s (Phoenix et al., 2006). Also, as pointed out by Ochoa-Hueso et al. (2011) much of the existing knowledge comes from ecosystems, where wet deposition (which occurs with precipitation) is dominant and where N deposition occurs at relative low rates and frequently throughout the year. This understanding might be of limited value for Mediterranean regions where high precipitation seasonality and cumulative dry deposition (occurring as aerosols or particles) most probably regulate ecosystem response to N deposition (Harpole et al., 2007; Bobbink et al., 2010; Ochoa-Hueso et al., 2011).

1.4 Grassland CO2 and water fluxes response to N enrichment

N addition has often been observed to enhance aboveground productivity by stimulating leaf photosynthesis and leaf area (e.g. Niu et al., 2010). Nevertheless, ecosystem CO_2 uptake through plant photosynthesis can be released back to the atmosphere via ecosystem respiration (Luo and Zhou, 2006), which can also be stimulated by N

enrichment (Luo and Zhou, 2006; Niu et al., 2010). How net ecosystem exchange (NEE) which represents ecosystem CO_2 balance (NEE = ecosystem photosynthesis + ecosystem respiration) responds to N is still uncertain. For instance, Bubier et al. (2007) found that for a bog ecosystem in Canada, increasing N addition lowered NEE and photosynthesis primarily due to loss of moss photosynthesis, but had little effect on ecosystem respiration. Conversely in a grassland dominated by *Bromus tectorum* L. in Nevada, N fertilization stimulated NEE (Cheng et al., 2009). Minor changes in CO_2 gas exchange were observed in a boreal mire over a 2-year period, but changes were depended on prevailing weather conditions (Saarnio et al., 2003). In fact, for a southern California grassland, Harpole et al. (2007) observed that N addition had no significant effect on NEE unless additional water was applied, reinforcing that Mediterranean systems have a strong water and N co-limitation. In Mediterranean grasslands, precipitation seasonality determines high inter-annual variation in CO_2 and water ecosystem fluxes (Huxman et al., 2004b, c; Pereira et al., 2007; Ma et al., 2007; Aires et al., 2008a,b; Jongen et al., 2011, 2014; Piayda et al., 2014). For instance, in a grassland dominated by C3 annuals in southern Portugal, NEE varied between −190 g CO_2 m^{-2} $year^{-1}$ (CO_2 sink) during a normal precipitation year and +49 g CO_2 m^{-2} $year^{-1}$ (CO_2 source) during a drought year (Pereira et al., 2007). Total evapotranspiration may also be substantially reduced during a dry year compared to a more favorable precipitation year (Aires et al., 2008, b). Indeed, these authors found a 24% decrease in evapotranspiration during a dry year. This is particularly important as grasslands play an important role in the exchange of water vapour and the atmosphere. For instance, in an evergreen oak woodland, the contribution of the herbaceous layer to total ecosystem transpiration can be as high as 44% (Paço et al., 2009). Although Mediterranean grassland productivity, is frequently co-limited by N and water (Hooper and Johnson, 1999; Harpole et al., 2007), studies addressing interactions between drought and N addition and its effects on grassland CO_2 and water exchange are scarce (Smith et al., 2016, but see Harpole et al., 2007 and García-Palacios et al., 2012). Particularly, there are few studies addressing the importance of extended summer/autumn drought on grassland productivity and CO_2 and water fluxes (see Ma et al., 2007; Miranda et al., 2009a; Jongen et al., 2011; Piayda et al., 2014) and considering interactions of drought and N deposition on Mediterranean grasslands.

1.5 Grassland response to multiple nutrients

Although, N is considered the element most often limiting plant production in terrestrial ecosystems (Vitousek and Howarth 1991), P rivals N in importance, as it plays a role in an array of processes such as energy generation, photosynthesis and N fixation. In fact, crop yield on 30–40% of the world's arable land is limited by P availability (Vance et al., 2003). Although P availability has also been increasing, the environmental consequences (e.g. decrease in plant species richness) have received less attention (DiTommaso and Aarssen, 1989; Ceulemans et al., 2014). Also, the importance of K to plant growth has been widely demonstrated (e.g. phloem transport, osmotic balance and photosynthesis), mainly in agricultural systems, however K has been far less studied in ecosystem ecology compared to other recognized critical nutrients (Kayser and Isselstein, 2005).

Although most studies have focused on limitation by N, many biomes, including grasslands, are frequently co-limited by multiple nutrients such as N and P (DiTommaso and Aarssen, 1989; Elser et al., 2007; Harpole et al., 2011; Fay et al., 2015). Indeed, in order to grow, species require multiple nutrients in particular ratios (i.e. nutrient stoichiometry) (Danger et al., 2008; Cleland and Harpole, 2010). For example, in regard to those nutrients that most often limit plant growth such as N and P (e.g. Elser et al., 2007; Fay et al., 2015), species may differ greatly in their underlying N:P stoichiometry (Vitousek et al., 2010; Cleland and Harpole, 2010; Ochoa-Hueso et al., 2011). Interestingly, Danger et al. (2008) found that competitive exclusion and coexistence mechanisms between species lead to an adjustment of community stoichiometry to that of their resources. Plant species coexistence and diversity is a result of habitat niche dimensionality (Harpole and Tilman, 2007; Harpole et al., 2016). More limiting resources (e.g. soil water, N and P) allow for the coexistence of several species, with a set of different characteristics, i.e. high trait divergence (Grime, 2006). Species diversity is thus maintained by functional trade-offs among species for below- and aboveground resources (Tilman, 1982). Thus, diverse communities are most probably limited by multiple nutrients (Cleland and Harpole, 2010). Nutrient enrichment reduces below-ground niche dimensionality, which reduces the number of species that can coexist (Tilman, 1982). Simultaneously, as nutrient addition increases biomass, light limitation may lead to species loss (e.g Tilman, 1987; Goldberg and Miller, 1990; Hautier et al., 2009). More recently DeMalach et al. (2017b) demonstrated that an increase in light asymmetry was the main mechanism behind the negative effect of nutrient enrichment on species richness. However, how decreased

multiple nutrient limitation will interact with precipitation variability to affect Mediterranean grassland productivity, diversity and stability remains poorly understood.

1.6 A Functional trait approach to monitor ecosystem responses

Taxonomic diversity, in particular species richness, has been commonly used to assess community responses to climate change (e.g. Tilman and El Haddi, 1992; Thuiller et al., 2005) or to nutrient availability (e.g. Stevens et al., 2010a, a; Flores-Moreno et al., 2016) and has frequently been related to ecosystem functioning (e.g. Tilman et al., 1996; 2014). However, global changes may induce changes in community composition which may not lead to a systematic loss of species (Dornelas et al., 2014), but might nevertheless affect ecosystem functioning (Mouillot et al., 2011). Alternatively, an increase in species richness does not always originate an increase in ecosystem functioning, as species may be functionally redundant (Cadotte et al., 2011). Nonetheless, the presence of functionally redundant species may ensure against the loss of ecosystem functioning following declines in species diversity (Yachi and Loreau, 1999; Cadotte et al., 2011). Species functional traits, defined as any measurable morphological, physiological or phenological feature of an individual (Díaz and Cabido, 2001) are considered to more accurately reflect species functional role in ecosystems and community responses to environmental factors or disturbances than taxonomical diversity (Tilman et al., 1997; Díaz and Cabido, 2001; Lavorel and Garnier, 2002; McGill et al. 2006; Violle et al., 2007; Cadotte et al., 2011; Mouillot et al., 2011).

Functional trait metrics, such as the community weighted mean (CWM) and functional diversity are commonly used to analyze community assembly and dynamics and may provide early signals of disturbance to ecosystems (e.g. Díaz et al., 1998; Mason et al., 2005; Grime, 2006; Garnier et al., 2007; Díaz et al., 2007; Cadotte et al., 2011; Mouillot et al., 2011; 2013). The CWM quantifies community functional structure. It is calculated for each individual trait and takes into account the relative abundance of the species carrying the trait, thus reflecting the dominant trait value in a community (Garnier et al., 2007; Violle et al., 2007; Díaz et al., 2007). The CWM is thus based on the “mass-ratio hypothesis”, that considers that the traits of the most abundant species are those that determine ecosystem processes (Grime, 1998). Functional diversity quantifies the distribution of trait values within the community. As taxonomical diversity, functional diversity may be

determined by different metrics: functional richness, evenness and divergence (e.g. Mason et al., 2005). However, functional divergence relates better to the functioning of ecosystems than functional richness or evenness (e.g. Mouillot et al., 2011). More recently, Laliberté and Legendre (2010) introduced a more robust functional divergence metric: functional dispersion, which is the mean distance in multidimensional trait space of individual species, weighted by their relative abundance, to the weighted centroid of all species.

1.7 Plant functional trait response to climate and nutrient addition

The high inter-annual precipitation variability in the Mediterranean climate (Lázaro et al., 2001), as the frequent occurrence of droughts (e.g. Lehner et al., 2006; Kovats et al., 2014), can modify the traits of the dominant species of grasslands, as well as the functional diversity within communities (Pérez-Camacho et al., 2012: Carmona et al., 2015; Rota et al., 2017). Drought may increase the relative abundance of small-sized species, species with a low specific leaf area (SLA) and high leaf dry matter content (LDMC) (e.g. Westoby et al., 2002; Pérez-Camacho et al., 2012; Carmona et al., 2015). Changes in leaf traits may have important consequences for ecosystem functioning, as they determine the rate of resource capture and utilization, as well as litter decomposition (Westoby et al., 2002; Lavorel and Garnier, 2002; Garnier et al., 2004). Drought may also favor species with an earlier onset and shorter flowering duration (Pérez-Camacho et al., 2012; Crimmins et al., 2013; Shavrukov et al., 2017) or increase the relative abundance of small-seeded species (Volis and Bohrer et al., 2013; Pérez-Camacho et al., 2012), thus affecting reproductive strategies.

The functional diversity of traits can also be affected by drought (Carmona et al., 2015; Rota et al., 2017). For instance, in a Mediterranean grassland, Rota et al. (2017) found that a drought year promoted the coexistence of species with different heights (high functional diversity in heights) whereas seed mass diversity was negatively affected, suggesting that different traits may respond differently to drought.

Nutrient availability is also a strong ecological filter that can determine community structure and functional trait diversity (Díaz et al., 1998; Grime, 2006). Nutrient addition may favor fast-growing species, which usually have a combination of high SLA, low LDMC, short-lived leaves and high stature, reflecting increased capability to acquire nutrients and light

quickly (Lavorel and Garnier, 2002; Suding et al., 2005; Ordoñez et al., 2009; Janeček et al., 2013; Zhou et al., 2016). Certain growth-forms such as grasses in detriment of rosette species can also be favored by nutrient addition (Zavaleta et al., 2003; Craine et al., 2001; Pfestorf et al., 2013). Species with N-fixing ability (e.g. legumes) may be favored by addition of P (e.g. DiTommaso and Aarssen, 1989; Sudding et al., 2005; Silvertown et al., 2006), whereas N addition may have a negative effect on legume abundance (van den Berge et al., 2011). Community phenology can also be disrupted by nutrient addition (Biederman et al., 2017). For example, Cleland et al. (2006) found that increased N addition delayed flowering in grasses and accelerated flowering in forb species. More importantly, nutrient addition and drought can select for different traits underlying opposite strategies. For example, drought may favor species with low SLA whereas nutrient addition may favor species with a high SLA. This draws attention to the need to understand possible interacting global change drivers.

A decrease in functional diversity with nutrient addition has been observed as increased competition for light resulted in increased trait convergence among co-existing species (Janeček et al. 2013; Helsen et al., 2014). However, others observed increased functional diversity (e.g. Niu et al., 2014) due to a higher niche differentiation among species which remained after nutrient addition, or did not detect changes in functional diversity (Li et al., 2015).

Communities with high functional diversity may have increased ecosystem functioning as a result of more efficient resource use (higher niche complementarity) (Mason et al., 2005; Díaz et al., 2007; Valencia-Gómez et al., 2015) and higher ecosystem resilience due to a broader range of strategies adopted by plants (Volaire et al., 2014; de la Riva et al., 2016).

Plant species in Mediterranean grasslands have evolved and adapted to cope with water and nutrient limitations (Noy-Meir, 1973; Peco et al., 1998). However, for many traits it is unclear how their CWM and functional diversity will respond to climate change predictions of increased precipitation variability and drought, nor is it clear how these communities will respond to decreased nutrient limitation. Also, community responses to nutrient addition may depend on water availability, as it is one of the primary drivers of ecosystem processes (Churkina and Running, 1998) especially in arid and semi-arid regions (Knapp and Smith, 2001; Huxman et al., 2004a, b, c). Water availability influences the diffusion of soil nutrients to plant roots and nutrient uptake (Cleland and Harpole, 2010). Thus, how

drought will interact with increased nutrient addition to impact community structure and functional diversity needs further understanding.

1.8 Aims of the thesis

The overall main objective of this thesis was to understand how nutrient addition and drought affect ecosystem structure and functioning of a semi-natural Mediterranean grassland dominated by annual species. In particular, the two first objectives were to assess the effects of extended summer/autumn drought and N deposition on: i) grassland productivity, species diversity, and phenology (Chapter 2), and ii) on CO_2 and water fluxes (Chapter 3). The third objective was to evaluate the effect of increasing number of added nutrients and inter-annual precipitation variability, as well as their interacting effects on: i) grassland species richness, ii) productivity and stability, and iii) grassland resistance, recovery and resilience to a disturbance event that consisted of intensive cattle grazing early in the growing season and a severe drought during winter and spring of the third year of the experiment (Chapter 4). The fourth and final objective, was to: i) assess changes in the functional structure (CWM) and functional diversity (functional dispersion, FDis) of the Mediterranean grassland, due to precipitation variability, increasing number of added nutrients and their interacting effects and ii) assess if CWM, FDis and species richness were related to grassland productivity (Chapter 5).

To meet the first two objectives, we established a manipulative greenhouse experiment using the seed bank of a semi-natural Mediterranean grassland dominated by annual species. We simulated three levels of extended summer/autumn drought (no drought, 50 days and 100 days of drought) and two levels of N deposition (no N and N addition) (at the rate projected for the Mediterranean Basin in 2050), through a factorial experimental design. To respond to objectives three and four, we conducted a nutrient addition field experiment, from 2013 to 2017, in the same semi-natural annual Mediterranean grassland where the seed bank, used to meet objectives one a two, was collected. We used N, P and K to establish three treatments of one, two and three added macronutrients, as well as control plots with no added nutrients (Harpole et al. 2016). In the field experiment, N was applied at high rates (100 kg ha^{-1} yr^{-1}) as proxy for chronic high N deposition and which is comparable to previous studies, whereas in the greenhouse experiment N was added at a

rate falling within the predicted N deposition scenarios for the Mediterranean Basin by 2050 (12 kg N ha^{-1} y^{-1}).

1.9 Study area

The study was conducted in a semi-natural Mediterranean grassland, at *Companhia das Lezírias*, an estate of approximately 15 000 ha, located north-east of Lisbon, Samora Correia, Portugal (38º49´45.13´´N, 8º47´28.61´´W).

The climate is Mediterranean, with mild, wet winters and hot, dry summers. Long-term (1961–1990) mean annual rainfall is 709 mm, with high inter annual variability. Mean long-term annual temperature is 15.9 °C, ranging from 10 ºC in January to 22.5 ºC in August (INMG, 1991). During the study period, from 2013 - 2017, the three consecutive hydrological years 2015, 2016 and 2017 (i. e. from October 1 to September 30) were dry years, with total precipitation being 465 mm, 598 mm and 483 mm respectively, that is 34%, 16% and 32% below the long-term mean annual precipitation respectively (Fig. 3). For the whole of mainland Portugal, 2015 and 2017 were classified as being extremely hot and dry, with 2017 being the second hottest and the third driest since 1931, all of which occurred after 2000 (IPMA, 20152017). The hydrological year 2013 was a normal precipitation year with 708 mm of total precipitation and 2014 was a wetter year with total precipitation 814 mm (Fig. 3).

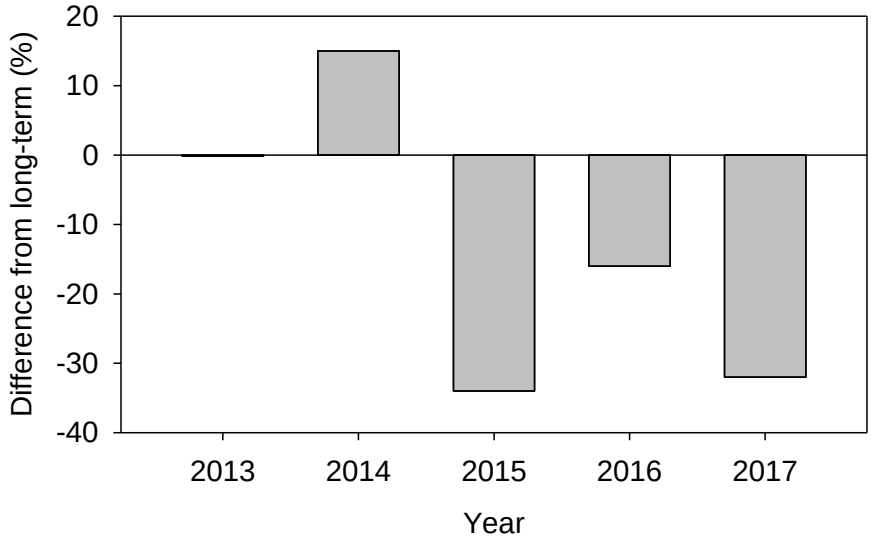

Fig. 3 Precipitation percentage difference to long-term annual mean (709 mm, 1961–1990).

Site topography is flat. The soil is a well-drained deep Haplic Arenosol (WRB, 2006) with a low water retention capacity and overall low nutrient availability (Table 1). Prior to treatment initiation, two soil cores (2.5 cm in diameter, 10 cm depth from soil surface) were collected from each plot. The plot subsamples were composited, homogenized, and air-dried. The Ecosystems Analysis Laboratory at the University of Nebraska assayed the soils to determine C (%) and N (%) using dry combustion GC analysis (COSTECH ESC 4010 Elemental Analyzer, Costech Analytical Technologies, Valencia, California, USA). Extractable soil P and K and soil pH were assayed at A&L Analytical Laboratory (Memphis, TN). Soil pH was measured using a 1:1 soil to water slurry.

Table 1 Pre-treatment nutrient soil content and pH values. Values refer to average of all plots (two soil cores per plot).

	Content
Carbon (%)	1.87
Nitrogen (%)	0.12
Phosphorus (ppm)	34.72
Potassium (ppm)	86.33
Calcium (ppm)	891.75
Magnesium (ppm)	54.03
Sulfur (ppm)	16.56
Sodium (ppm)	26.11
Boron (ppm)	0.66
Copper (ppm)	1.57
Iron (ppm)	463.08
Manganese (ppm)	23.94
Zinc (ppm)	6.13
pH	5.93

The grassland community, occasionally grazed by cattle until 2012, is dominantly composed of annual C3 species, which emerge after first autumn rains and senesce and set seed in late spring. Despite inter-annual changes in species composition, dominant forb species are *Tolpis barbata* L., *Plantago bellardii* All. and *Tuberaria guttata* (L.) Fourr., dominant grasses are *Agrostis pourretii* Willd., *Avena barbata* Link and *Gaudinia fragilis*

(L.) P.Beauv., and dominant legumes are *Ornithopus compressus* L., *Trifolium arvense* L. and *Trifolium campestre* Schreb.

1.10 Experimental design - *Greenhouse experiment*

In a greenhouse at the School of Agriculture, University of Lisbon, Portugal, we conducted a pot experiment using the top soil seed bank from the semi-natural Mediterranean grassland at *Companhia das Lezírias* (Fig. 4a, b), between October 2014 and May 2015. To assess the effect of an extended summer/autumn drought and N deposition on the grassland community, we imposed three levels of extended drought: no drought, 50 days (moderate) and 100 days of drought (severe), crossed in a factorial design with two levels of N deposition: no N addition (i.e. controls) and N addition, for a total of 6 treatments. Each treatment had 8 replicates (a total of 48 plots). N was added in the form of ammonium nitrate (NH_4NO_3) at the rate of 12 kg N ha^{-1} y^{-1} which is the average projected rate of atmospheric N deposition in the Mediterranean Basin for 2050 (Phoenix et al., 2006).

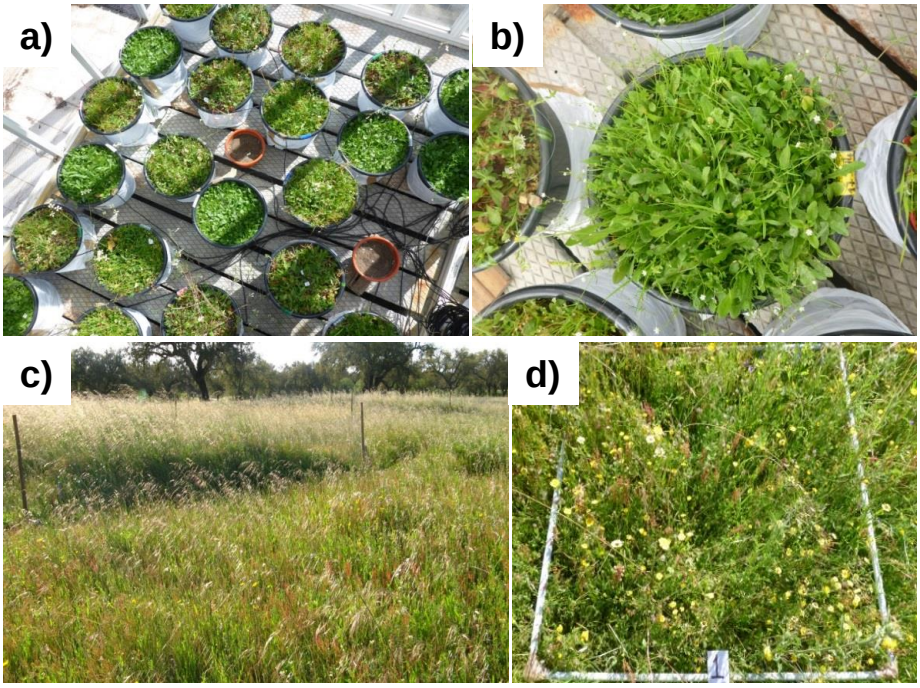

Fig 4 a) Greenhouse experiment. b) Detail of one of the pots in the greenhouse experiment (control pot with 50 days watering delay). c) Field experiment, at the back (taller vegetation) NPK enriched plot, at the front N enriched plot. d) Species composition quadrat (1x1 m), in early May, on a plot enriched with PK. Photographs by C. Nogueira.

1.11 Experimental design - Field experiment

In June 2012, a completely randomized block design with 3 blocks, 8 treatments per block, and 3 to 6 replicates per treatment was established following the general protocol of the Nutrient Network (www.NutNet.org). Each plot was 5 x 5 m, separated by 1-m walkways. Plots were subdivided into four, 2.5 x 2.5 m subplots (Fig. 5). These 4 subplots were randomly assigned to be used for core sampling and other studies (e.g. functional traits). The core sampling subplot was further divided into 4 (1x1 m) subplots, surrounded by a 0.25 m buffer. One of these 1-m^2 subplots was randomly designated for plant species composition and leaf area index measurements and the other three for aboveground biomass (Fig. 4 c, d and Fig. 5)

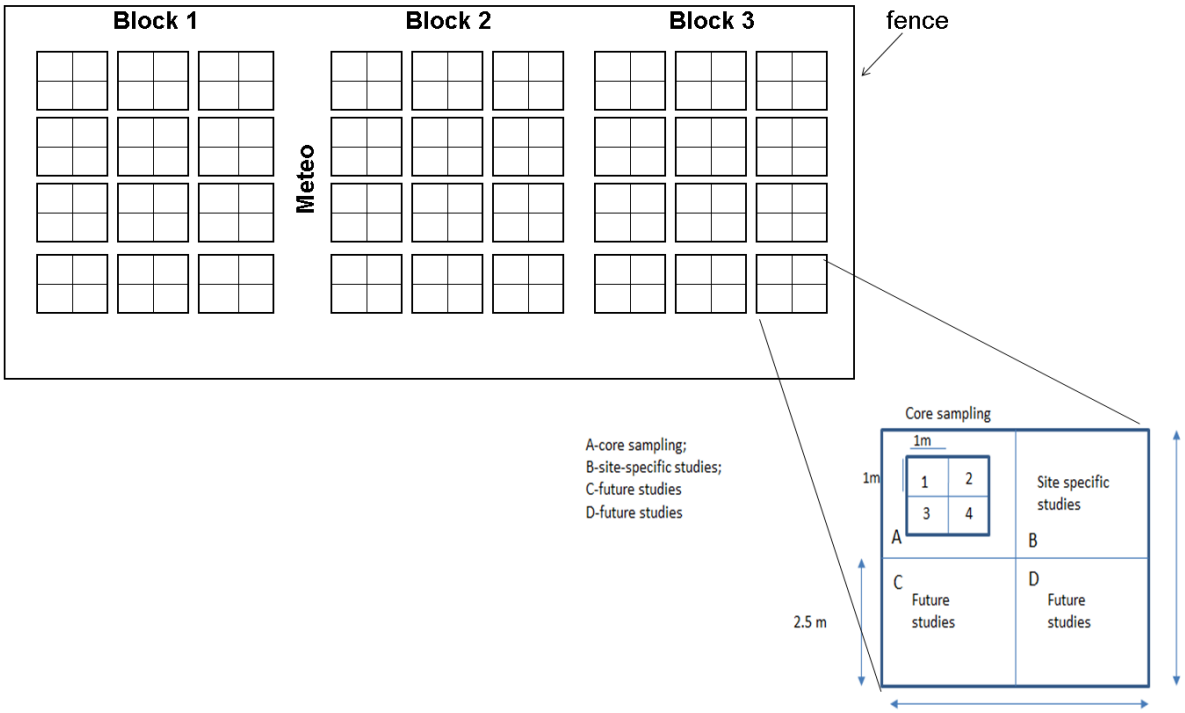

Fig. 5 Design layout for the field experiment based on the NutNet protocol. The three blocks are represented and also the location of the weather station (Meteo). Adapted from: http://www.nutnet.umn.edu.

To assess multiple resource limitation, each nutrient (N, P and K) was applied with two levels (control, added), and crossed in a factorial design. Nutrients were applied every fall

(i.e. at the beginning of the growing season) from 2012 until 2017. N was added from 2012 to 2016 as slow-release urea (60-90 days) at a rate of 10 g Nm^{-2} yr^{-1}, switching in 2017 to two separate additions of urea (3 months apart to mimic slow release) due to restricted availability of timed-release urea. P was added as triple-super phosphate and K as potassium sulfate, both at a rate of 10 g m^{-2} yr^{-1}. Micronutrients (6% Ca, 3% Mg, 12% S, 0.1% B, 1% Cu, 17% Fe, 2.5% Mn, 0.05% Mo and 1% Zn) were added at a rate of 100 g m^{-2} with K only in the first year to avoid possible micronutrient toxicity.

The site was fenced to avoid cattle grazing and trampling. At the end of each growing season and after seed production and dispersal, all standing vegetation in the fenced experimental area was cut to approximately 5 cm aboveground, with a brush cutter and removed, leaving only short stubbles and roots. This was done to eliminate potential effects of carried-over biomass such as a decrease in forb richness and dominance of high biomass species do to litter accumulation (Dudney et al., 2017).

We conducted continuous measurements of air temperature, relative humidity (VP-3 humidity and temperature sensor, Decagon Devices, Pullman, USA) and photosynthetic photon flux (QSO-S PAR Photon Flux sensor, Decagon Devices, Pullman, USA), recorded and logged every 30 min (EM50 data logger, Decagon Devices, Pullman, USA). Precipitation was also recorded (tipping bucket RG2, Delta-T Devices, Cambridge, UK) by connection to a logger (CR1000 data logger, Campbell Scientific, Logan, USA).

1.12 The Nutrient Network – NutNet

Our field experimental site is included in the Nutrient Network (NutNet, www.nutnet.org) (Borer et al., 2017). NutNet is a coordinated experiment, following a standardized protocol to supply nutrients, exclude large-herbivores and collect data. NutNet was designed to improve our understanding of productivity-diversity relationships under widely varying biotic and abiotic conditions. The network comprises 80 sites that follow complete NutNet setup, in total there are more than 100 sites. These sites include a wide range of herbaceous-dominated ecosystems (i.e. ranging from arctic to desert grasslands) (Fig. 6).

In appendix 1, as an example of the NutNet work, we present a manuscript entitled “Leaf nutrient contents but not specific leaf area increase rapidly and predictably in response to eutrophication” from a collaborative work of 27 NutNet sites, which includes the

Portuguese site at *Companhia das Lezírias* and for which we provided data and contributed to the writing of the manuscript. The result of other collaborational work is the manuscript entitled “On estimating Gross Primary Productivity of Mediterranean grasslands under different fertilization regimes using vegetation indices and hyperspectral reflectance”, also presented in appendix (appendix 2).

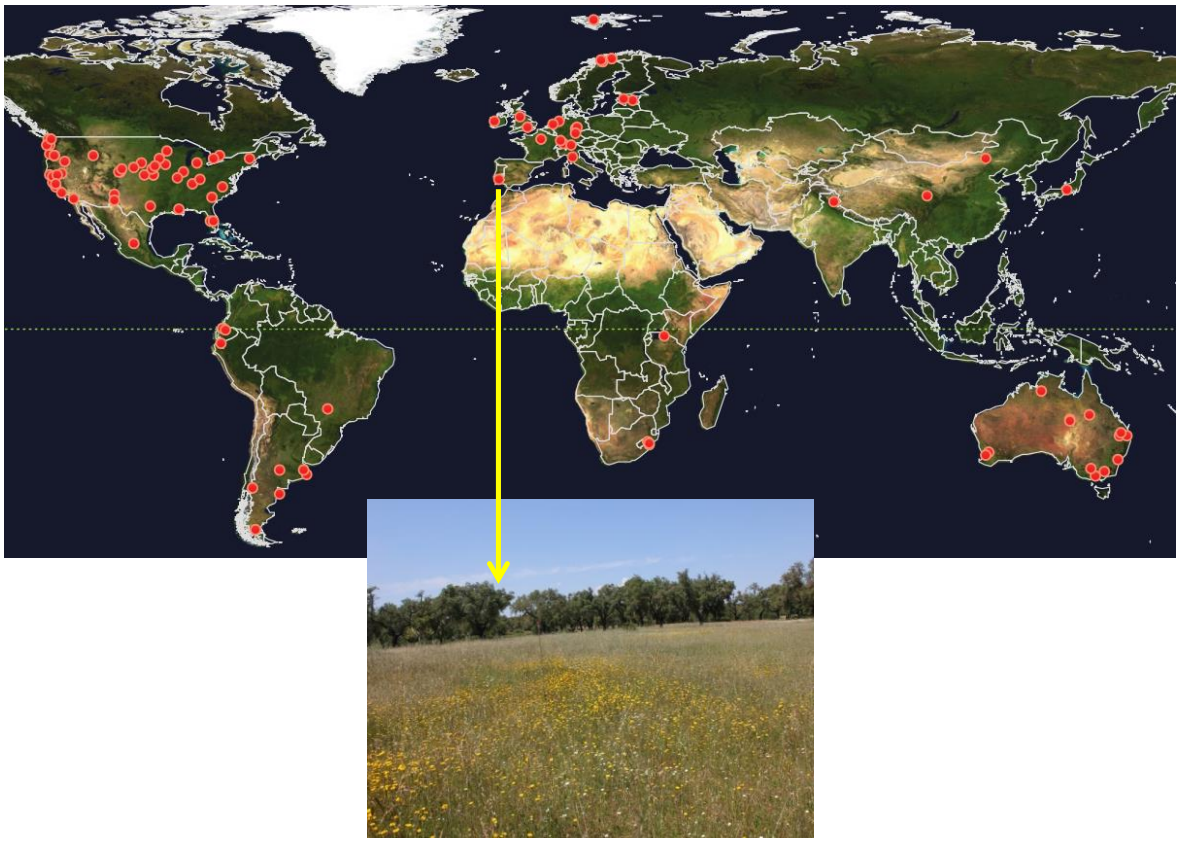

Fig. 6 NutNet participating sites. Red points denote location of the more than 100 sites participating in the NutNet project. Source: Borer et al. (2017). Photograph: General view of the Portuguese site at *Companhia das Lezírias* (M.C. Caldeira).

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CHAPTER 2

Extended autumn drought, but not nitrogen deposition, affects the diversity and productivity of a Mediterranean grassland

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2 Extended autumn drought, but not nitrogen deposition, affects the diversity and productivity of a Mediterranean grassland

2.1 Abstract

Global change is affecting ecosystems worldwide. Nitrogen (N) deposition is increasing globally and climate change scenarios forecast an increase of prolonged autumn droughts particularly in the Mediterranean regions. Mediterranean grasslands occupy 1.3 million km^2 of the terrestrial surface, host high biodiversity values and generate important ecosystem services. Understanding the effects of N deposition and prolonged autumn drought on the productivity and dynamics of Mediterranean grasslands is crucial to support the conservation of these ecosystems in a global change context.

We assessed the effects of extended autumn drought and N deposition on the productivity, species diversity, and phenology of a Mediterranean grassland through a manipulative, greenhouse, experiment. We simulated three levels of extended drought: no drought, 50 days and 100 days of drought and two levels of N deposition: no N and N addition, through a factorial design experiment. Severe drought (100 days) affected negatively grassland productivity, through a reduction of the growing season duration and accumulated growing degree days. Moderate drought (50 days) affected productivity negatively but productivity values recovered to levels similar to those of the non-drought treatment.

Severe and moderate drought delayed plant phenology decreasing significantly the number of individuals with mature flowers and with fruits or seeds. Plant functional groups responded differently to drought with productivity of forbs, but not productivity of graminoids and legumes, being negatively affected by moderate and severe drought. N addition did not affect grassland productivity, diversity or phenology.

Our results anticipate effects of climate change on the productivity and species composition of Mediterranean grasslands.

Keywords: diversity; drought; Mediterranean grassland; nitrogen deposition; phenology; productivity.

2.2 Introduction

Humans are causing significant global environmental change, including climate change and nitrogen (N) deposition (Smith et al., 2009; Phoenix et al., 2006; Suddick et al., 2013). Global and regional model simulations project, for semi-arid and arid regions, increased temperatures and higher frequency of more prolonged droughts (Miranda et al., 2002; Lehner et al., 2006; Kovats et al., 2014). N deposition has also been increasing mainly due to fossil fuel combustion and agricultural fertilization (Galloway et al., 2004; Peñuelas et al., 2012). These changes can alter plant resource availability, biodiversity and ecosystem functioning (Fay et al., 2000).

The Mediterranean Basin (Myers et al., 2000), which climate is characterized by dry and hot summers and cool and wet winters, is located in a climate change hotspot (Giorgi, 2006; Costa et al., 2012). Increased duration of summer/autumn dry spells is expected to occur in this region. The Mediterranean region is also considered a biodiversity hotspot hosting several ecosystems of conservation value, including grasslands (Myers et al., 2000). Mediterranean grasslands, excluding cultural grasslands, occupy approximately 1.3 million km2 of the terrestrial surface (Dixon et al., 2014), support high biodiversity and provide essential ecosystem services, including forage for wild and domestic animals, soil protection, water and climate regulation or wildfire prevention (Joffre et al., 1999; Hector and Bagchi, 2007; Bugalho and Abreu, 2008).

Assessing the effects of autumn drought spells and N deposition on plant diversity and productivity of Mediterranean grasslands is of critical interest for understanding their ecological functioning in the context of global environmental change.

Mediterranean grasslands are characterized by a typically high diversity of annual plant species (Pitt and Heady, 1978; Díaz-Villa et al., 2003) which complete their life cycle within one growing season. In these ecosystems, plant productivity and species composition is tightly coupled to timing, frequency and amount of rainfall (Espigares and Peco, 1993, 1995; Peco and Espigares, 1994). When water is available, plant growth is strongly dependent on occurrence of accumulated optimal temperatures (Peñuelas and Filella, 2001) and co-limited by availability of nutrients, such as N (Hooper and Johnson, 1999; Harpole et al., 2007; Báez et al., 2007). Plants start growing with first autumn rain events (Pitt and Heady, 1978; Peco, 1989) and most of the primary production occurs during spring (Moreno and Pulido, 2009), lasts until late spring and early summer when plants set

seed and senesce. Delayed onset of autumn precipitation may strongly affect species and functional group composition, as well as plant phenology, ultimately determining grassland diversity and productivity (Espigares and Peco, 1993; Walck et al., 2011). Most grassland studies around the world have focused on the responses of primary productivity and species composition to rainfall variability, mainly in spring growing season, through manipulation of total precipitation (e.g. Fay et al., 2000; Knapp et al., 2002; Jongen et al., 2013). Very few studies however, addressed the effects of extended autumn drought on grassland diversity and productivity (but see Espigares and Peco, 1995; Miranda et al., 2009).

N deposition can also affect productivity, and plant species composition of Mediterranean grasslands (Báez et al., 2007; Bobbink et al., 2010; Stevens et al., 2010). Results however, are contradictory and dependent on N deposition levels. Within a global change context, 12 kg $ha^{-1}year^{-1}$ of N deposition have been forecasted for 2050 in the Mediterranean Basin (Phoenix et al., 2006). Such level of N deposition is generally much lower than levels added in most grassland experiments (e.g. Zavaleta et al., 2003; Dukes et al., 2005; Clark et al., 2007; Smith et al., 2016), which vary between 50 and 100 kg $ha^{-1}year^{-1}$. There is indeed a dearth of data on the effects of N deposition, at levels forecasted for the Mediterranean region, on the ecology of Mediterranean grasslands (Ochoa-Hueso et al., 2011).

Here, through a manipulative greenhouse experiment, we assess the effects of prolonged autumn drought and N deposition on productivity, plant species composition and plant phenology of a semi-natural Mediterranean grassland. We tested the effects of 3 levels of prolonged autumn drought and 2 levels of N deposition on grassland communities artificially established in experimental pots. We hypothesize that: 1) extended autumn drought will decrease plant productivity but N addition will attenuate this effect; 2) extended drought and N addition will both reduce plant diversity; 3) extended drought will retard plant phenology by shortening plant growing season; 4) biomass, diversity and phenology of plant functional groups will respond differently to extended drought and N addition.

2.3 Materials and Methods

Study site and experimental setup

The study was conducted in a greenhouse at the School of Agriculture, University of Lisbon, Portugal (38^{o}42´27.5´´N, 9^{o}10´56.3´´W) between the 14th of October 2014 and the 15th of May 2015. We used top soil seed bank of a semi-natural Mediterranean grassland at Companhia das Lezírias, an estate of approximately 15 000 ha, located north-east of Lisbon, Portugal (38^{o}49´45.13´´N, 8^{o}47´28.61´´W). The grassland community, occasionally grazed by cattle, is dominantly composed by annual species which emerge after first autumn rain events and senesce and set seed in late spring. Plant species composition has been measured in the study site during the last 4 years under an ongoing long-term experiment - Portuguese site of the Nutrient Network experiment (www.nutnet.umn.edu; Seabloom et al., 2013). Dominant forbs are *Tolpis barbata* L., *Plantago bellardii* All. and *Tuberaria guttata* (L.) Fourr., dominant grasses are *Agrostis pourretii* Willd., *Avena barbata* and *Gaudinia fragilis* (L.) P.Beauv., and dominant legumes are *Ornithopus compressus* L., *Trifolium arvense* L. and *Trifolium campestre* Schreb. Aboveground biomass is 403.24 g m^{-2} ± 35.47 (mean ± s.e.m.) and species richness 22.66 ± 1.36 (Nogueira et al. unpublished results). The climate is Mediterranean, with mild, wet winters and hot, dry summers (Caldeira et al., 2001). Mean annual temperature is 14.9°C. Mean annual precipitation is 537 mm falling mainly between October and April. Site topography is flat. The soil is a well-drained deep Haplic Arenosol (IUSS, 2006) with a low water retention capacity and low total N content (0.18 mg N kg^{-1} dry soil) (Nogueira et al. unpublished results).

The soil seed bank was collected in mid-September by randomly locating and sampling 35 quadrats (20 x 50 cm) in the semi-natural grassland. In each quadrat the uppermost 3 cm of the soil surface was collected (Luzuriaga et al., 2005). Soil samples were homogenized, sieved through a 3 mm mesh to remove excessive litter and rocks and air-dried at room temperature for 48 h and kept in the laboratory.

Forty-eight PVC pots (29.5 cm inner diameter and 24 cm height) perforated at the bottom, for water to drain freely, were lined with a gardening screen cloth to avoid soil loss. To avoid heating, the containers were wrapped with a white plastic film. Pots were filled with homogenized and roughly sieved dry soil from the grassland site.

Top soil (1000 cm3) was spread on top of each pot forming a thin layer (15 mm) of homogenized soil containing the seed bank of the sampled grassland community. To assess the effect of an extended autumn drought and N deposition on the grassland community, we imposed 3 levels of extended drought: no drought, 50 days (moderate) and 100 days of drought (severe). These water delay treatments were crossed in a factorial design with 2 levels of N deposition: no addition (i.e. control) and N addition, for a total of 6 treatments. Each treatment had 8 replicates. Thus, complete treatments were: (1) C0–no drought (watering of pots started on October the 14th); (2) C50 – 50 days of drought (watering started on December the 3rd); (3) C100 – 100 days drought (watering started on January the 20th); (4) N0 – no drought plus N addition; (5) N50 – 50 days drought plus N addition; (6) N100 – 100 days of drought plus N addition.

N was added in the form of ammonium nitrate (NH4NO3) at the rate of 12 kg N ha^{-1} y^{-1} which is the average projected rate of atmospheric N deposition in the Mediterranean Basin for 2050 (Phoenix et al., 2006).

Irrigation was done twice a week and soil was maintained moist during the whole experiment. N fertilizer was added to distilled water given to the N addition treatments, and the control pots received distilled water without fertilizer.

Pots were randomly placed in the greenhouse and positions changed frequently. Light attenuation was 16%, calculated as the difference between light values measured outside and inside the greenhouse. Measurements where done with a ceptometer (AccuPAR model LP-80, Decagon Devices, Pullman, USA). Relative humidity and air temperature in the greenhouse were logged every 30 min (VP-3 humidity temperature and vapor pressure sensor; EM50 data logger, Decagon Devices, Pullman, USA) (Fig. S1). Volumetric soil water content was continuously measured using EC-5 soil moisture sensors (Decagon Devices, Pullman, USA) installed in three pots per treatment. Average soil water content, after irrigation started and throughout the remainder of the experiment, was 22.93% ±1.41, 21% ±1.15 and 23.88% ± 2.23 for no drought, moderate and severe drought respectively. To control for external seed contamination, we have set up in the greenhouse eight control pots filled with sterilized soil that were randomly arranged and watered regularly. No seed contamination was observed during the experiment.

Measurements

On April 15th and May 15th, 2015, four randomly chosen replicates of each treatment were destructively sampled for assessing plant biomass, species composition and phenology (n = 4, total of 24 pots at each sampling date). Mid- to late April is generally the peak of vegetation growth in Mediterranean grasslands, coinciding with warm temperature and water availability (Lázaro et al., 2001) whilst in mid-May, most species start to senesce with rapidly raising air temperatures. We determined for all communities: 1) the total number of individual plants per species; 2) the number of individuals per species with flower buds (all stems with buds and no mature flowers present); 3) the number of individuals per species with mature flowers; and 4) the number of individuals per species with fruits or seeds. Individuals were cut at ground level and live biomass was separated from senescent biomass and recorded for each species. All biomass was oven dried at 60 ºC for 48 h prior to weighing.

Community growth rate (CGR) from germination (t_1) to sampling date in May (t_2) was calculated as:

$$CGR = \frac{biom2 - biom1}{t2 - t1},$$

where $biom_1$ is community biomass at germination (considered as zero) and $biom_2$ is community biomass at time t_2.

The average plant species individual weight was estimated as the ratio of the total weight of that species in the community and the number of individuals of that species in the community. Plant density of each community was reported as the sum of all individual plants in each pot.

We calculated species richness (R), Shannon's diversity (H0) and Simpson's dominance index (D) in April and May 2015 (Magurran and McGill, 2011). These indexes were estimated in each pot and per functional group (non-legume forbs, herein defined as forbs, legumes and graminoids) in each pot. Species richness was estimated as the total number of plant species per pot. Diversity was calculated using Shannon's diversity index,

$$H' = -\sum_{i=1}^{S} pi\ ln\ (pi),$$

where S is the total number of species in each plot and pi is the proportion of total biomass contributed by each species. Dominance was calculated using Simpson's dominance index,

$$D = \sum_{i=1}^{S} pi^2$$

Plant growth and phenology depend on accumulated temperatures (e.g. Peñuelas and Filella, 2001), that we have calculated as the growing-degree days (GDD) index. Growing-degree days index assumes that plant growth occurs above a threshold temperature (Tb) and that plant growth and phenology are positively related to accumulation of mean daily temperature above that temperature threshold. Growing-degree day was calculated as:

$$GDD = \left(\frac{Tmax - Tmin}{2}\right) - Tb,$$

where Tmin is daily minimum air temperature, Tmax the daily maximum air temperature, and Tb is the base temperature, 5.5 ºC, i.e., the temperature below which the rate of development is considered to be insignificant (Spinoni et al., 2015). For mean daily temperatures below Tb, growing-degree days was considered 0; for Tmin values below 5.5 ºC for a given day Tmin was considered as 5.5 ºC; for Tmax values above 30 ºC for a given day, Tmax was set to 30 ºC. The accumulated growing-degree days is the sum of the growing-degree days between germination date and biomass harvesting (April 15th or May 15th).

Data analysis

A three-way ANOVA, with drought levels, N addition and sampling month as factors, was used to analyze aboveground biomass, plant diversity (R, H' and D) and plant phenology (total number of individuals; number of individuals with flower buds; number of individuals with mature flowers and number of individuals with fruits or seeds). When a significant difference was found for a main factor, a Tukey or Holm-Sidak pairwise multiple comparison procedures was applied to determine individual differences between means. Pearson correlation, with n = 48, was used to relate: diversity and productivity, diversity and plant density of each community and accumulated growing- degree days with plant

phenology. Dependent variables were tested for homogeneity of variances and normality and trans- formed to meet these criteria when necessary. Level of significance was P = 0.05 for all tests. Statistical analysis was performed with Sigmaplot 13.0 (Systac Software, Inc., San Jose, USA).

2.4 Results

Total aboveground biomass

Overall, aboveground biomass decreased significantly with increasing drought ($P < 0.001$) and increased between April and May ($P < 0.001$). However, there was a significant drought and month interaction ($P < 0.001$) (Fig. 1A) as in April, total aboveground biomass was significantly different between all levels of drought ($P < 0.001$, for all comparisons), but in May, aboveground biomass of the no drought and moderate drought treatment were similar and significantly higher compared to the severe drought ($P = 0.001$ and $P = 0.010$, respectively) (Fig. 1A). Moreover, whilst biomass did not change significantly between April and May in the no drought treatment, it increased significantly by 17% ($P = 0.005$) and 42% ($P < 0.001$) in the moderate and extreme drought, respectively. The percentage of senescent biomass declined significantly ($F = 73.06$; $P < 0.001$) with increasing drought and was significantly higher in May than in April ($F = 44.49$; $P < 0.001$). There were no effects of N addition on biomass production (Fig. 1A).

Accumulated growing-degree days decreased with extending drought treatments (Fig. S2). Additionally, aboveground biomass was positively correlated with accumulated growing-degree days ($r = 0.77$, $P < 0.001$). Community growth rate increased significantly ($F = 8.82$, $P < 0.001$) with increasing drought (2.12 g m^{-2} d^{-1}± 0.10; 2.68 g m^{-2} d^{-1} ± 0.12; 3.17 g m^{-2} d^{-1} ± 0.11, for no drought, moderate, extreme drought, respectively).

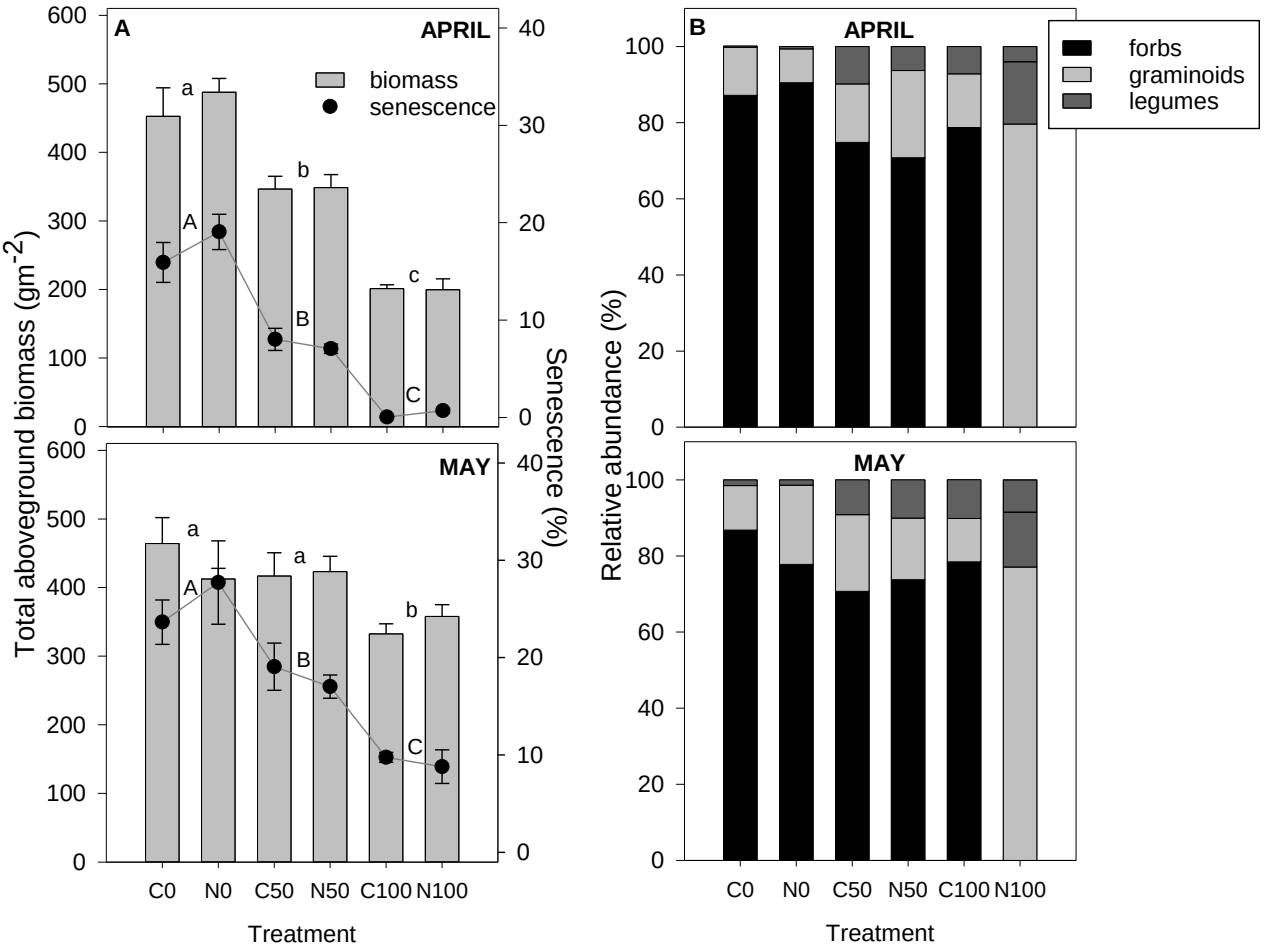

Fig. 1 A. Total aboveground biomass (bars) and percentage of senescent biomass (lines) in April and May (mean ± SE, *n* = 4). Different letters indicate significantly different means between drought treatments (Tukey's post hoc test, $P < 0.05$); small letters refer to differences in total aboveground biomass and capital letters refer to differences in percentage of senescent biomass. B. Proportion of biomass of different functional groups in drought and N addition treatments in April and May. C – no added N (control), N – N addition and the number next to the letters represent the number of days of extended drought.

Aboveground biomass of functional groups

Aboveground biomass was dominated by forbs (78.85% ± 1.11, mean ± s.e.m, across all treatments), followed by grasses (15.42% ± 0.86) and legumes (5.74 % ± 0.68). *Silene gallica* (26.80% ± 0.02), *Tolpis barbata* (14.23 % ± 0.01) and *Raphanus raphanistrum* (15.42% ± 0.02) were the dominant forb species, *Agrostis pourretii* (9.35% ± 0.01) and

Vulpia ciliata (2.12% ± 0.003) prevailed within grass species and *Ornithopus compressus* was the most abundant legume species (4.17% ± 0.01) (Table S1).

Relative abundance of functional groups was significantly affected by the drought treatments ($P < 0.001$ for forbs and legumes and $P = 0.010$ for graminoids) (Fig. 1B). Abundance of forbs was significantly higher and that of legumes significantly lower, in the no drought treatment both in April and May ($P < 0.001$) (Fig. 1B). Between April and May, the abundance of legumes ($P = 0.005$), but not of forbs ($P = 0.066$), increased significantly with drought (Fig. 1B). Abundance of graminoids did not differ between April and May, and was significantly higher in the moderate drought compared to the other drought treatments (no drought: $P = 0.017$; extreme: $P = 0.025$) (Fig. 1B).

There were no significant effects of N addition on the abundance of functional groups.

Species richness, diversity and dominance

Species richness and diversity were significantly lower ($F = 36.78$, $P < 0.001$; $F = 89.25$, $P < 0.001$, respectively) and dominance significantly higher ($F = 50.58$, $P < 0.001$) in the no drought treatment, both in April and May (Figs. 2, S3). Species richness, diversity and dominance were not significantly different, in April or May, between the moderate and extreme drought treatments (Figs. 2, S3). Species richness increased significantly ($F = 4.27$, $P = 0.022$) between April and May in communities subjected to the extreme drought treatment (Fig. S3). Total aboveground biomass related negatively with diversity ($r = -0.55$, $P < 0.001$). There were no significant effects of N addition on species richness, diversity and dominance.

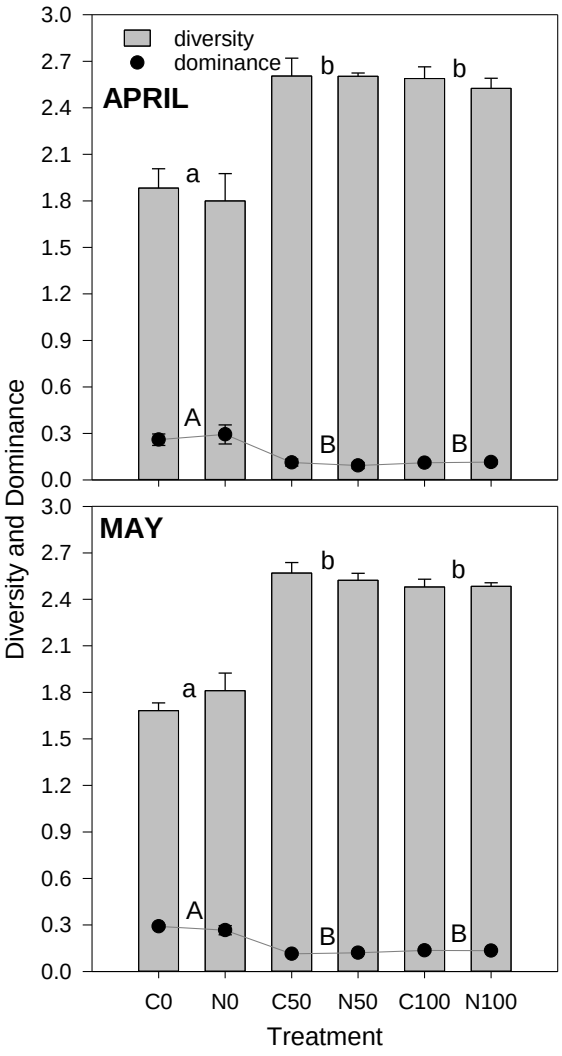

Fig. 2 Community Shannon diversity (bars) and Simpson dominance (lines) indexes, in drought and N addition treatments in April and May. Mean ± SE, n = 4. Different letters indicate significantly different means between drought treatments (Tukey's post hoc test, $P < 0.05$); small letters refer to differences in diversity and capital letters refer to differences in dominance. C – no added N (control), N – N addition and the number next to the letters represent the number of days of extended drought.

There were, on average for all treatments, 19.38 ± 0.42, 5.00 ± 0.20 and 4.31 ± 0.23 species per pot of forbs, graminoids and legumes, respectively. Forbs was the more species-rich and diverse functional group (Figs. 3, S3). Species richness and diversity of forbs were significantly lower ($F = 21.48$, $P < 0.001$; $F = 51.01$, $P < 0.001$, respectively) and dominance significantly higher ($F = 38.60$, $P < 0.001$) in the no drought treatment (Figs. 3, S3).

Richness and diversity of graminoids increased significantly ($F = 27.07$, $P < 0.001$; $F = 42.70$, $P < 0.001$, respectively), and dominance decreased significantly ($F = 39.382$, $P < 0.001$) with increasing drought (Figs. 3, S3). Furthermore, graminoid species richness and diversity decreased significantly ($F = 7.84$, $P = 0.008$; $F = 5.99$, $P = 0.019$, respectively) between April and May (Figs. 3, S3).

Legume diversity was significantly lower (F = 3.46, P = 0.042) in the no drought compared to the extreme drought, but similar to the moderate drought treatment (Fig. 3). However, drought did not affect legume dominance (Fig. 3). Legume species richness was significantly lower (F = 6.35, P = 0.004) in the no drought treatment in May (Fig. S3).

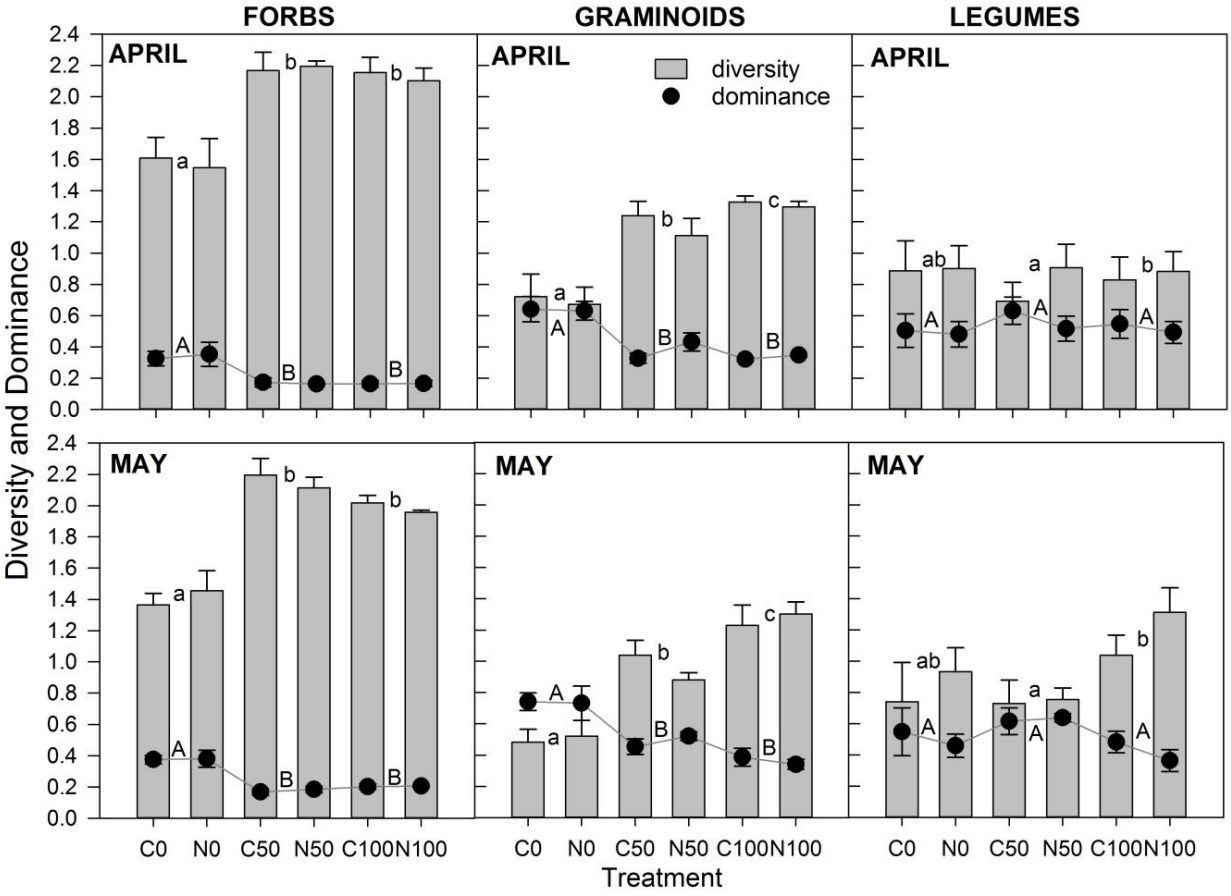

Fig. 3 Shannon diversity (bars) and Simpson dominance (lines) indexes for each functional group, in drought and N addition treatments in April and May. Mean ± SE, n = 4. Different letters indicate significantly different means between drought treatments (Tukey's post hoc test, P < 0.05); small letters refer to differences in diversity and capital letters refer to differences in dominance. C – no added N (control), N – N addition and the number next to the letters represent the number of days of extended drought.

Plant density and individual plant species weight

Plant density was significantly lower (F = 99.91, P < 0.001) in the no drought treatment and there were no significant differences between the moderate and extreme drought treatments (Fig. S4).

Average individual plant weight within species was significantly higher in the no drought treatment (F = 9.35, P < 0.001; Fig. S5) but was not affected by N addition or harvesting date.

In April, average individual plant weight in each functional group was significantly different between drought treatments (F = 6.11, P = 0.004) and functional groups (F = 89.15, P < 0.001), with a significant interaction between drought and functional group (F = 8.38, P < 0.001). N addition did not have a significant effect on average individual plant weight. Individual plant weight of forbs was on average 87.16%, 73.98% and 68.64% significantly higher (P < 0.001) than plant weight of the other functional groups, for the no drought, moderate and severe drought treatments, respectively. In the no drought treatment, average individual plant weight of graminoids was significantly higher than of legumes (P < 0.001). For the moderate and severe drought treatments there were no significant differences in individual plant weight between graminoids and legumes. In May, drought and N addition did not have a significant effect on average individual plant weight per functional group. However, average individual plant weight was significantly different between functional groups (F = 60.97, P < 0.001).

Species richness and diversity were positively (r = 0.77, P < 0.001; r = 0.82, P < 0.001, respectively), and dominance negatively (r = 0.76, P < 0.001) correlated with plant density.

Plant phenology

The proportion of individuals in vegetative state increased significantly, and that with mature flowers and fruits or seeds, decreased significantly with increasing drought (Table 1, Fig. 4). Also, as expected, there was a significantly higher proportion of individuals in vegetative state, and with buds and a lower proportion of individuals with mature flowers and fruits or seeds, in April than in May (Table 1, Fig. 4).

Table 1 Statistical results for the three-way-Anova for percentage of individuals in vegetative state, with flower buds, mature flowers, fruits or/and seeds for drought, N addition treatments and sampling month. *DF* (degrees of freedom); *F* values from the statistic Anova; *P* values <0.05 were considered significant. Percentage of individuals in a vegetative state and percentage of individuals with fruits and/or seed was square root transformed. *P* values in bold mark significant differences.

		Vegetative		Buds		Flowers		Fruits/seeds	
	DF	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Extended drought	2	75.36	**<0.001**	12.12	**<0.001**	94.64	**<0.001**	69.89	**<0.001**
N addition	1	0.30	0.589	0.20	0.661	0.65	0.426	0.52	0.476
Month	1	387.30	**<0.001**	24.44	**<0.001**	318.67	**<0.001**	133.17	**<0.001**
Extended drought x N addition	2	0.56	0.576	0.36	0.703	0.98	0.386	0.05	0.952
Extended drought x month	2	4.01	**0.027**	14.85	**<0.001**	2.80	0.074	0.39	0.683
N addition x month	1	0.40	0.53	0.10	0.751	0.09	0.773	0.80	0.378

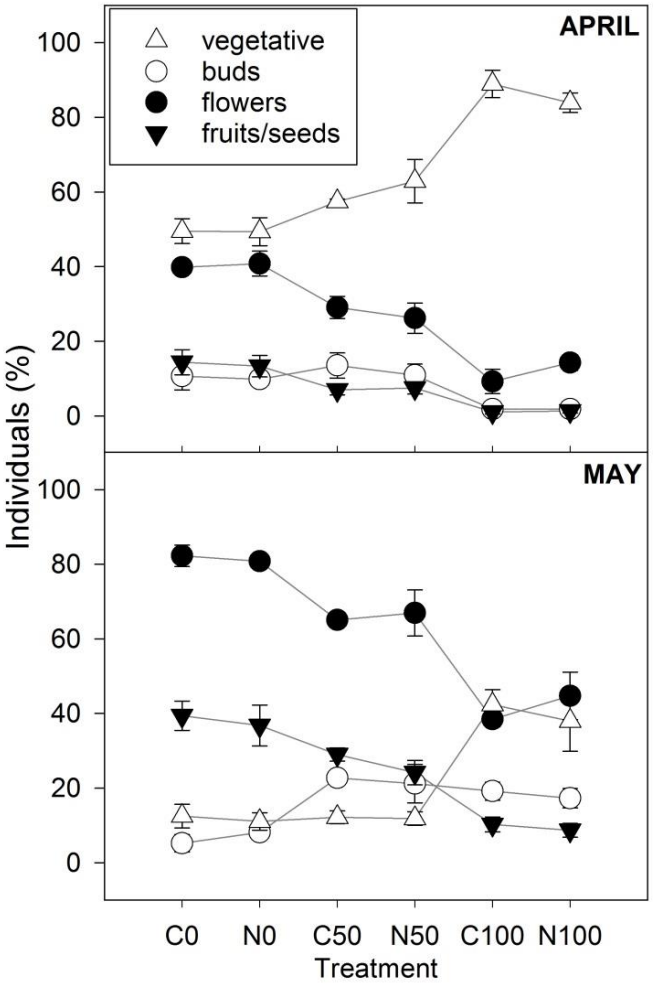

Fig. 4 Percentage of individuals in the community in the phenological stages of vegetative, flower bud, mature flowers and fruits and/or seeds, in April and May, in drought and N addition treatments in April and May. Mean ± SE, *n* = 4. C – no added N (control), N – N addition and the number next to the letters represent the number of days of extended drought. Grey lines along phenological states are to enable better reading.

The phenological maturity of forbs (that is, the number of flowering and fruiting or seeding individuals) decreased significantly with increasing drought in May (F = 104.44, P < 0.001; F = 98.13, P < 0.001, respectively) (Fig. 5). Also, for forbs, the proportion of vegetative individuals was significantly higher in the extreme drought treatment (F = 66.72, P < 0.001).

Proportion of flowering and fruiting/seeding of graminoids was only significantly lower (F = 11.75, P < 0.001; F = 9.65, P < 0.001, respectively) in the severe drought treatment. There were only graminoids in the vegetative state in the extreme drought treatment and none were present in the other drought treatments. There were no significant differences in the phenology of graminoids, between the no drought and moderate drought treatments.

Legumes had higher proportion of individuals with mature flowers in the moderate drought treatment (F = 13.92; P < 0.001). The proportion of fruiting/seeding legume individuals in the no drought treatment was significantly higher than in the severe drought but not different from the moderate drought treatment (F = 13.55; P < 0.001) (Fig. 5).

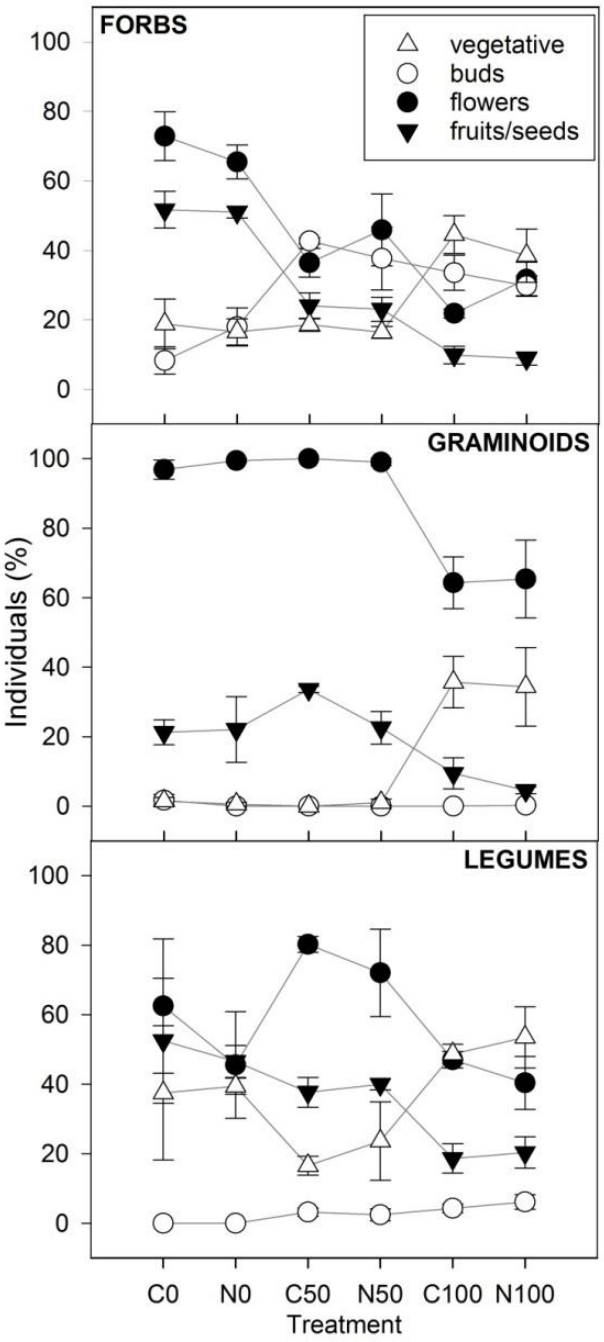

Fig. 5 Percentage of individuals of each functional group (forbs, graminoids and legumes) in the phenological stages of vegetative, flower bud, mature flowers and fruits and/or seeds in drought and N addition treatments in May. Mean ± SE, *n* = 4. C – no added N (control), N – N addition and the number next to the letters represent the number of days of extended drought. Grey lines along phenological stages are to enable better reading.

For all functional groups, the proportion of flowering and fruiting/seeding individuals was positively related with accumulated growing-degree days (Fig. 6), although stronger for forbs than for legumes and graminoids (Fig. 6).

N addition did not have a significant effect on community or functional group phenology (Table 1, Fig. 5).

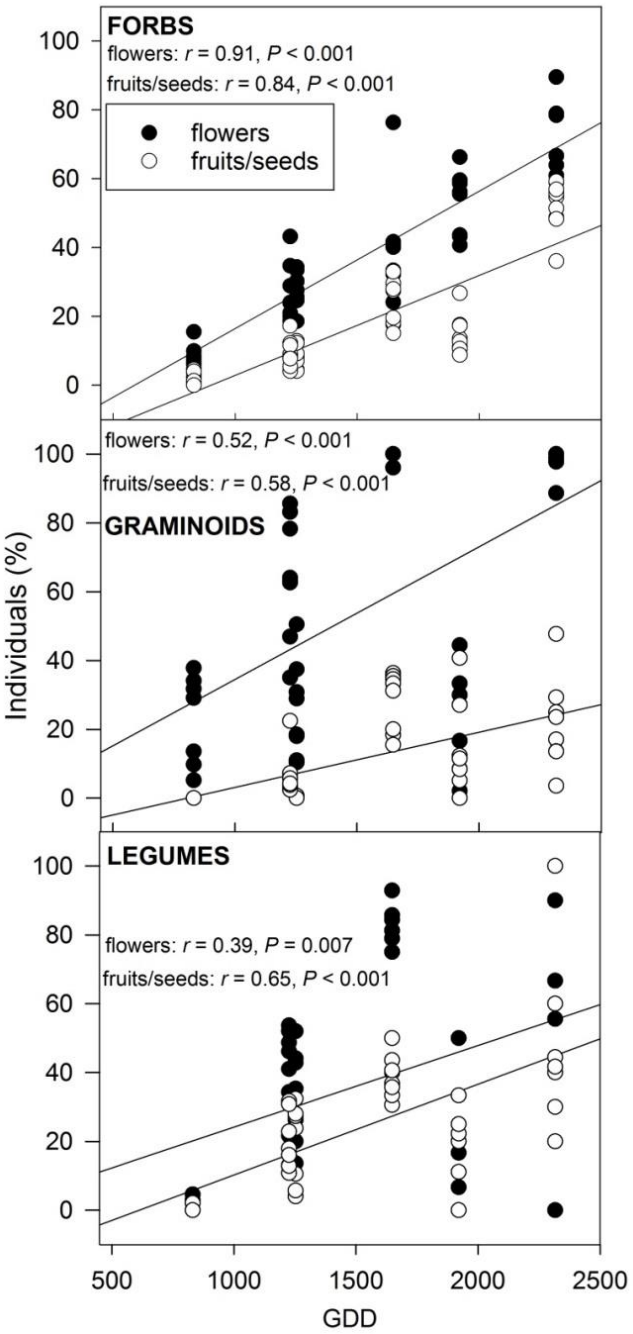

Fig. 6 Relationships between accumulated GDD and percentage of individuals with flowers and percentage of individual with fruits and/or seeds for each functional group (forbs, graminoids and legumes (*n* = 48)). Significant relationships were determined from Pearson's correlation coefficients.

2.5 Discussion

Extended autumn drought decreased grassland productivity and delayed plant phenology. This may have been due to a reduction of the plants' growing period and a decrease of accumulated growing-degree days caused by the delay of first autumn rain events. Additionally, extended drought affected grassland diversity positively most likely because

of lower competitive exclusion effects by dominant species. Finally, and unexpectedly, N addition did not affect grassland productivity and diversity.

Effects of extended drought on grassland productivity and diversity

The lower productivity of grassland communities at extended droughts reflects lower effective plant growth (i.e. the period during which there was no water limitation) in these communities. Concomitantly, senescence also declined significantly with increasing drought, reflecting lower accumulated growing-degree days (Gan and Amasino, 1997).

Decreased Mediterranean grassland productivity at the end of the growing season, when subjected to autumn drought was also observed by other authors (e.g. Cabello et al., 2012; Miranda et al., 2009). Our results emphasize the ecological importance of early autumn rain events in determining productivity of Mediterranean grasslands (Lázaro et al., 2001).

Grassland communities under moderate drought revealed high resilience, as aboveground biomass increased to similar levels as those for the no drought treatment. However, in the communities subjected to severe drought although there was also a noteworthy increment in aboveground biomass, it remained lower than that of the other drought treatments, suggesting a lower resilience. Average community growth rate was 26.30% and 49.53% higher in the moderate and severe drought treatments, respectively, than in the no drought treatment, explaining the productivity recovery of these communities. Indeed, younger communities tend to have higher relative growth rates (Poorter, 1989).

Decreased aboveground biomass production with increasing drought resulted mainly from the lower biomass of forbs, the dominant functional group in the grassland communities. Functional groups, however, responded differently to drought treatments. Biomass of legumes related positively with moderate and severe drought whilst that of graminoids was negatively affected by severe drought. Timing of first autumn rain events is the main determinant between-year variation in the species composition in Mediterranean grasslands (Espigares and Peco, 1995; Peco and Espigares, 1994; Peco et al., 1998). Such variation is caused by disproportionate responses of plant functional groups (Corbin et al., 2007; Báez et al., 2013). For example, legumes are usually favored by late autumn rain events (Pitt and Heady, 1978) and may present a high-temperature dormancy, even

when seeds imbibe water, as a protection in the case of false break (Del Pozo and Aronson, 1999). This might also explain the low legume biomass in the no drought treatment (84.75% and 81.68% lower than the moderate and severe drought, respectively, in May). In fact, in the no drought treatment, seeds were exposed to high air temperatures (average maximum temperatures ~30 ºC) when compared to temperatures of 21 ºC and 20 ºC, in the moderate and extreme drought treatments, respectively. This is a pivotal point, because with predicted global change scenarios of extended autumn droughts, the environmental context and ecological constraints faced by grassland communities will also change (Dukes et al., 2005; Smith et al., 2009). In our experiment, this was demonstrated by the differences of accumulated growing-degree days among drought treatments and the significant positive relationship between accumulated growing-degree days and plant productivity and phenology (Peñuelas and Fillela, 2001; Menzel, 2003).

Forbs were the dominant functional group in all communities. Being richer in species, forbs probably also included a wider representation of a range of germinating and growing conditions, including species favored by early or later autumn rain events. Still, forbs seemed to be at advantage, as compared to other functional groups, in the communities subjected to no drought, where they had the higher proportion of biomass and individuals with higher weight. Early autumn rain events favor larger and higher biomass plants (Espigares and Peco, 1993). Several of the forb species that were present in our communities (e.g., *Echium plantagineum*, *Raphanus raphanistrum*) included large individuals (in this case rosette plants), with high cover and biomass, which corroborates higher dominance and lower diversity indexes recorded in the communities with no drought. Graminoids were the most resilient group responding to drought treatments. In fact, only the severe drought affected negatively graminoid biomass in April, and by May differences between drought treatments were attenuated. Also, although biomass production increased significantly between April and May, the proportion of graminoids remained similar between the two sampling dates. This agrees with other studies showing that graminoids are not strongly affected by precipitation variability (Zavaleta et al., 2003).

Increased drought affected positively grassland diversity, although drought has often been associated with reduced plant species richness and diversity (Tilman and El Haddi, 1992; Copeland et al., 2016). The significant negative relationship between community diversity and total aboveground biomass, suggests that competitive interactions shaped our communities (Knapp et al., 2002; Baer et al., 2003). Early autumn rain events enabled the

establishment of species with high plant cover and biomass that may have outcompeted some species or prevented the posterior establishment of other species which may explain the lower plant density in the no drought treatment. Thus, the lower diversity in the no drought treatment (as compared to the higher diversity in the drought treatments) may have not been only a direct response to the extended drought but also a result of the higher individual biomass of established species.

Effects of extended drought on plant phenology

Increased drought delayed plant phenology. Phenological stages (e.g. flowering, seeding) are strongly determined by a threshold of accumulated temperatures (i.e. growing-degree days) (Peñuelas and Fillela, 2001; Menzel, 2003). Lower accumulated growing-degree days in the severe drought was reflected in 39.95% ± 3.32 and 28.68% ± 1.30 less individuals with flowers and fruits or seeds than those individuals in the no drought treatment. Delayed effects of drought on phenology were also observed for other Mediterranean ecosystems such as forests and shrublands (Peñuelas et al., 2004; Miranda et al. 2009). Phenological responses however, varied across functional groups. Phenological delay with drought, particularly in forbs, the dominant functional group in the investigated grasslands, may strongly affect future overall species composition. Conversely, graminoids and legumes showed some phenological plasticity to moderate and extended drought, as no significant differences were observed between these drought treatments. These results suggest that novel patterns of species composition may arise in Mediterranean grasslands due to differential rainfall-mediated phenological changes among functional groups (Peñuelas et al., 2004; Miranda et al., 2009; Dorji et al., 2013).

Nitrogen effects

N addition did not increase aboveground biomass production nor did it drive changes in plant species composition or phenology. Many experimental studies found a positive relationship between N addition and plant productivity and a negative relationship between N addition and species richness (e.g.; Zavaleta et al., 2003; Suding et al., 2005; Clark et al., 2007). In most of these studies, however, the amount of N addition was in excess of plant demand or at rates higher than those of natural N deposition rates (Cleland and

Harpole, 2010; Ochoa-Hueso et al., 2011). Also, most of the research on the impacts of N deposition on Mediterranean ecosystems has been conducted in California, USA, with less focus on the Mediterranean Basin (Bobbink et al., 2010; Ochoa-Hueso et al., 2011, 2013). Some of these latter studies have shown, as a result of N input, increased plant productivity and no effects or decreased species richness in grassland ecosystems (Bonanomi et al., 2006, 2013).

N is generally a limiting nutrient (Vitousek and Howarth, 1991), as is in the investigated grassland, due to low soil N (Spehn et al., 2002; Nogueira et al. unpublished results) and low estimated background deposition (ca. 5.55 kg N ha^{-1} yr^{-1}; http://www.emep.int/http://www.emep.int/). Moreover, the low percentage of biomass of legumes (ca. 6%) should not significantly drive N pools as it has been found for the same study site by Spehn et al. (2002). As such, we were expecting increased biomass production and decreased species richness with N deposition, due to, for example, changes in competition balances or soil acidification (Stevens et al., 2010). Other factors may contribute to explain the lack of N effects in our experiment. N deposition is, indeed, a proxy of long-term cumulative N deposition and thus, the relationship between N deposition and community composition and richness may have been developed for long (Caporn et al., 2016). In this way, the N addition rate used in our study and the length of our experiment (one growing season) may not have been enough to assess N effects on productivity and species composition. Also, co-limitation by other nutrients such as phosphorus (P) (Elser et al., 2007); which is a limiting nutrient in our grassland (Nogueira et al., unpublished results) and other Mediterranean ecosystems (Ochoa-Hueso and Stevens, 2015), may have impaired grassland responses to N addition. In a recent study across a wide range of climates, soil conditions and vegetation types in the United States, Simkin et al. (2016) found that the negative relationships between N deposition and species richness are indeed common, but not universal, and that fine-scale processes mediated by soil and climate variables, can moderate vegetation responses to N deposition. Although we did not observe a response at the community and functional group level there may have been positive or negative species-specific responses to N deposition such as reported in other studies (Ochoa-Hueso and Stevens, 2015).

2.6 Conclusions

Extreme events, such as extended autumn droughts, are forecasted to increase in Mediterranean regions, under climate change scenarios, which will affect the productivity and species composition of grasslands as shown in our study. Such changes may also have important legacy effects such as a decreased seed bank density and richness (Ladwig et al., 2012; Dudney et al., 2017). Because we only address one growing season in this work we could not assess the effects of extended autumn drought, and N deposition, on next seed generation (e.g. seed mass). This is an important limitation of our study that remains to be investigated in the future. Our results also show a delay of plant phenology with extended drought which may affect ecological functioning and resilience of these grasslands under a global change context (Gordo and Sanz, 2005; Llorens and Penuelas, 2005; Miranda et al., 2009; Zeppel et al., 2014).

Changes in plant productivity, diversity and phenology may potentially affect the ecological functioning and, ultimately, ecosystem service delivery of Mediterranean grasslands. Understanding the ecological mechanisms affecting Mediterranean grasslands under global change scenarios will contribute to better support management practices targeting the conservation of these ecosystems.

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2.8 Supplementary data

Table S1 List of plant species present in our experiment for each functional group.

Forb functional group	Graminoid functional group	Legume functional group
Anagallis arvensis	*Agrostis pourretii*	*Lathyrus angulatus*
Andryala integrifolia **	*Avena barbata*	*Ornithopus compressus*
Bellardia trixago	*Briza maxima*	*Ornithopus pinnatus*
Cerastium glomeratum	*Briza minor*	*Trifolium glomeratum*
Cerastium sp	*Bromus hordeaceus*	*Trifolium angustifolium*
Chamaemelum mixtum	*Bromus madritensis*	*Trifolium arvense*
Coleostephus myconis	*Gaudinia fragilis*	*Trifolium campestre*
Crepis capillaris	*Juncus bufonius*	*Trifolium ligusticum*
Crepis vesicaria	*Juncus capitatus*	*Trifolium resupinatum*
*Echium plantagineum***	*Poa* sp	
Erodium sp	*Vulpia ciliata*	
Galium sp	*Vulpia geniculata*	
Geranium molle		
Hedypnois cretica		
Hypochaeris glabra		
*Leontodon taraxacoides***		
*Linum bienne***		
Misopates orontium		
Orobanche minor		
Parentucellia viscosa		
Petrorhagia nanteuilii		
Plantago bellardii		
*Plantago coronopus**		
Plantago lagopus		
Raphanus raphanistrum		
*Rumex acetosella***		
Rumex bucephalophorus		
Silene colorata		
Silene gallica		
Spergula arvensis		
Stachys arvensis		
Tolpis barbata		
Tuberaria guttata		

Species without (*) are annual species
*Annual or biannual species
** Perennial species

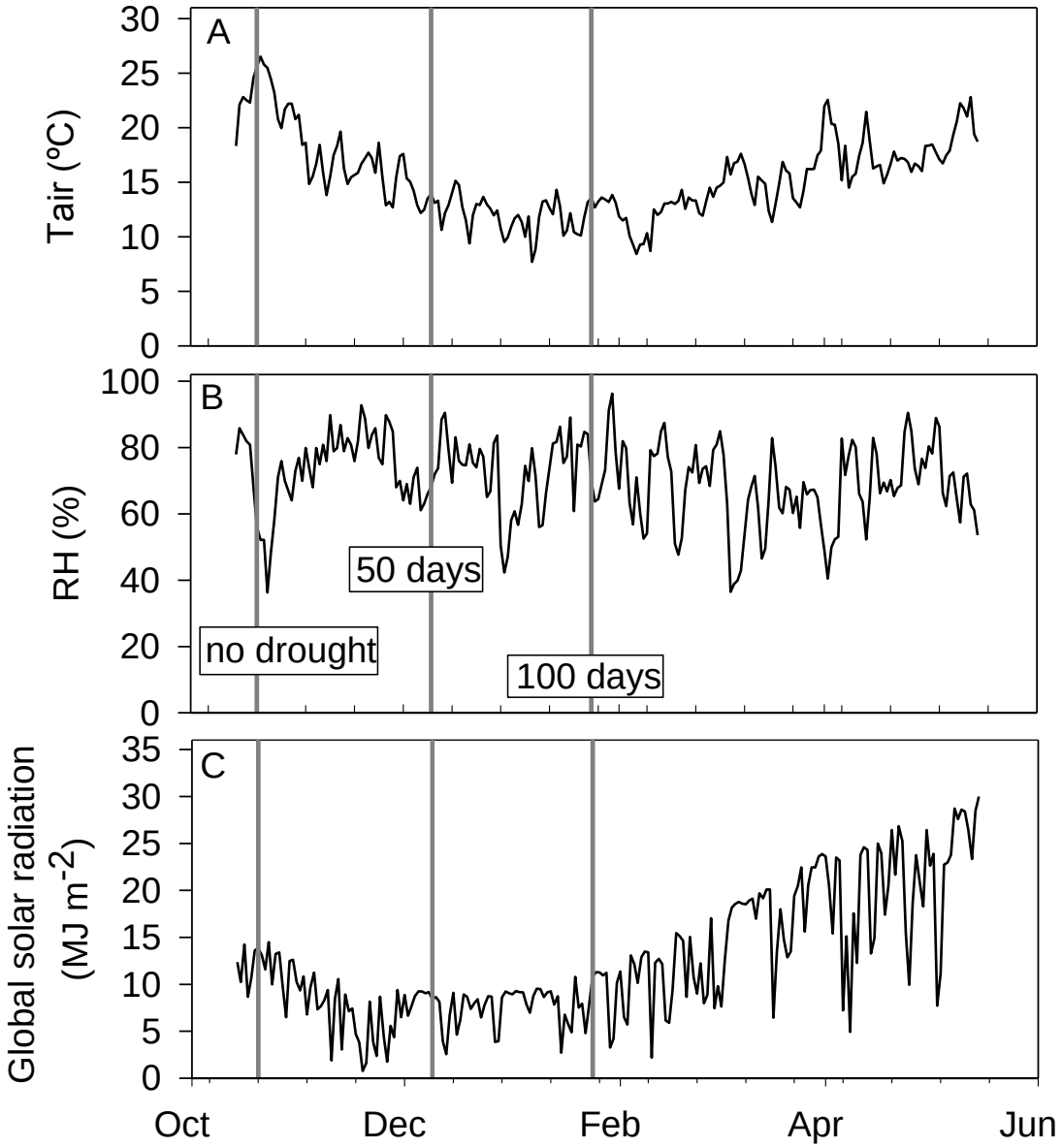

Fig. S1 A. Daily average air temperature (ºC); B. Daily average relative humidity (%) and C. Total daily global solar radiation (MJm^{-2}) over the course of the study. Vertical lines indicate date of germination.

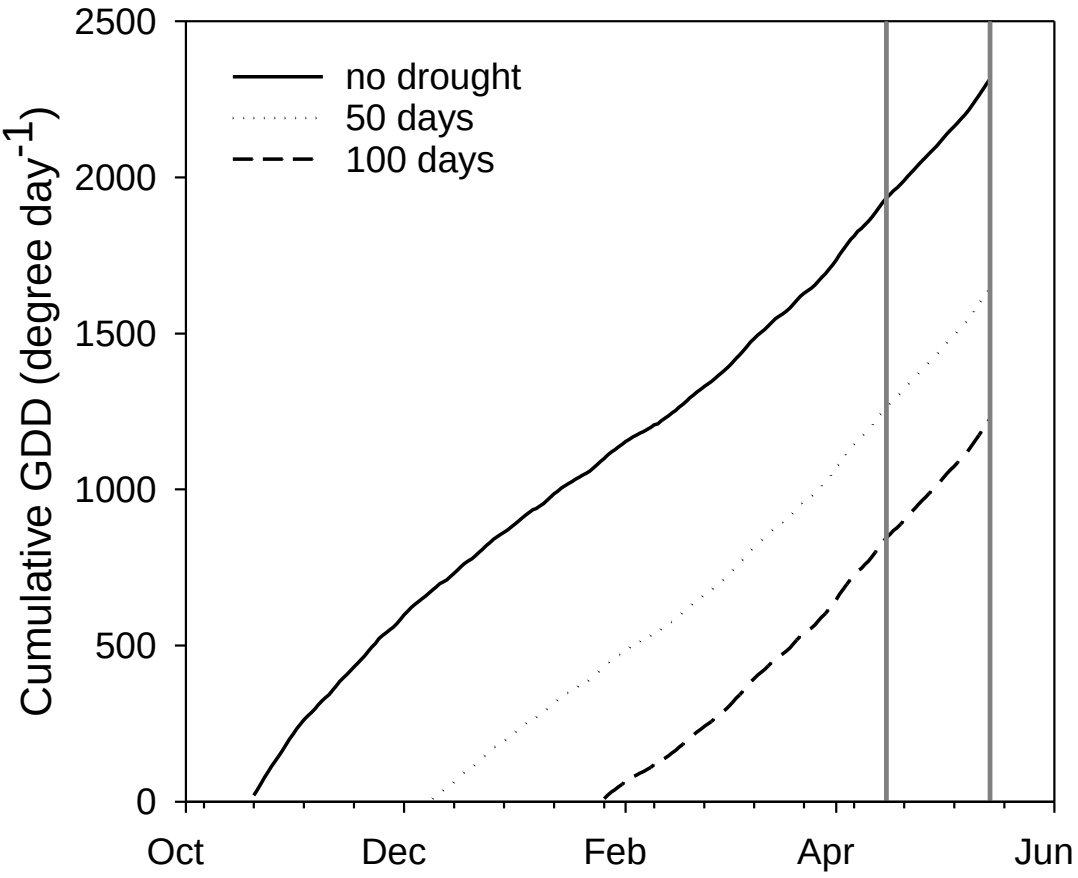

Fig. S2 Accumulated GDD (Tb = 5.5ºC) from germination to April and May sampling dates (grey vertical references lines) for each drought treatment (no drought; 50 days drought and 100 days drought).

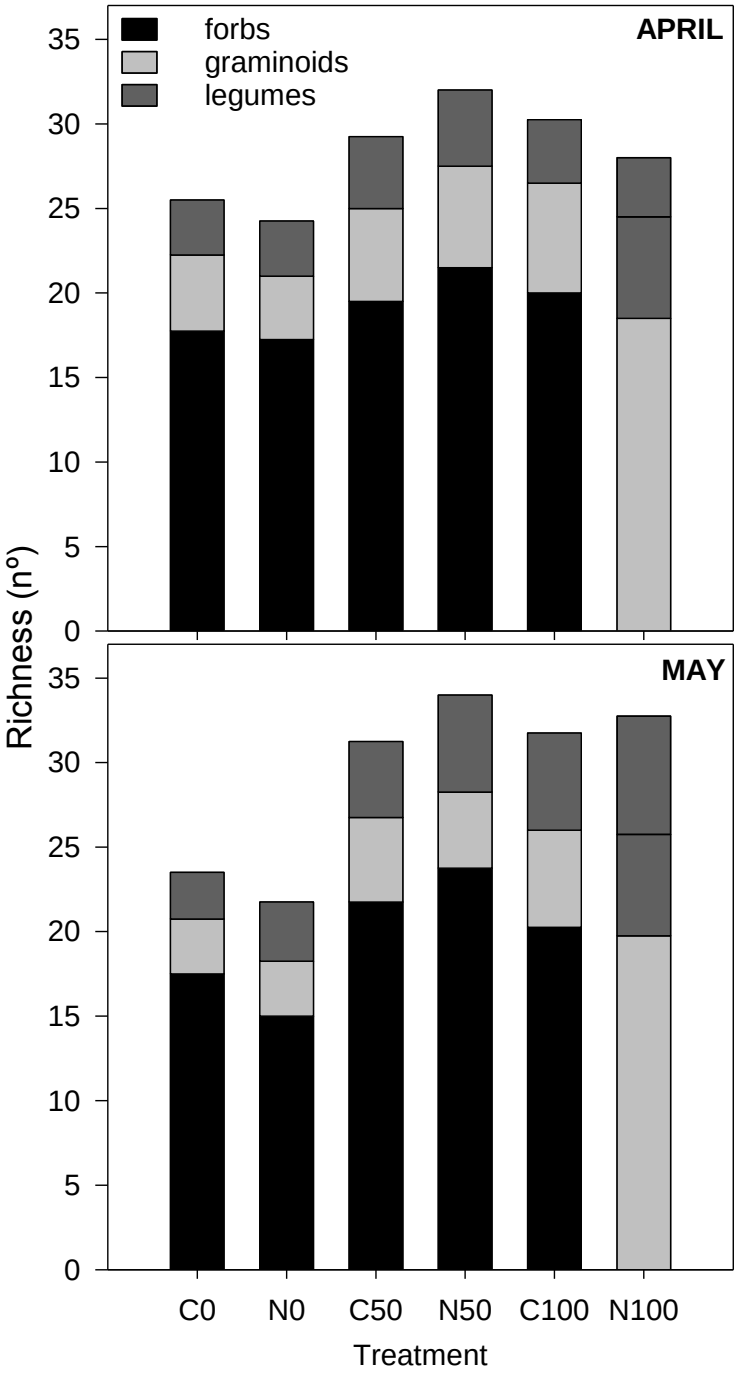

Fig. S3 Average species richness for each functional group, in drought and N addition treatments in April and May. C - no added N (control), N - N addition and the number next to the letters represent the number of days of extended drought. Significant differences between treatments are not presented for graph clarity.

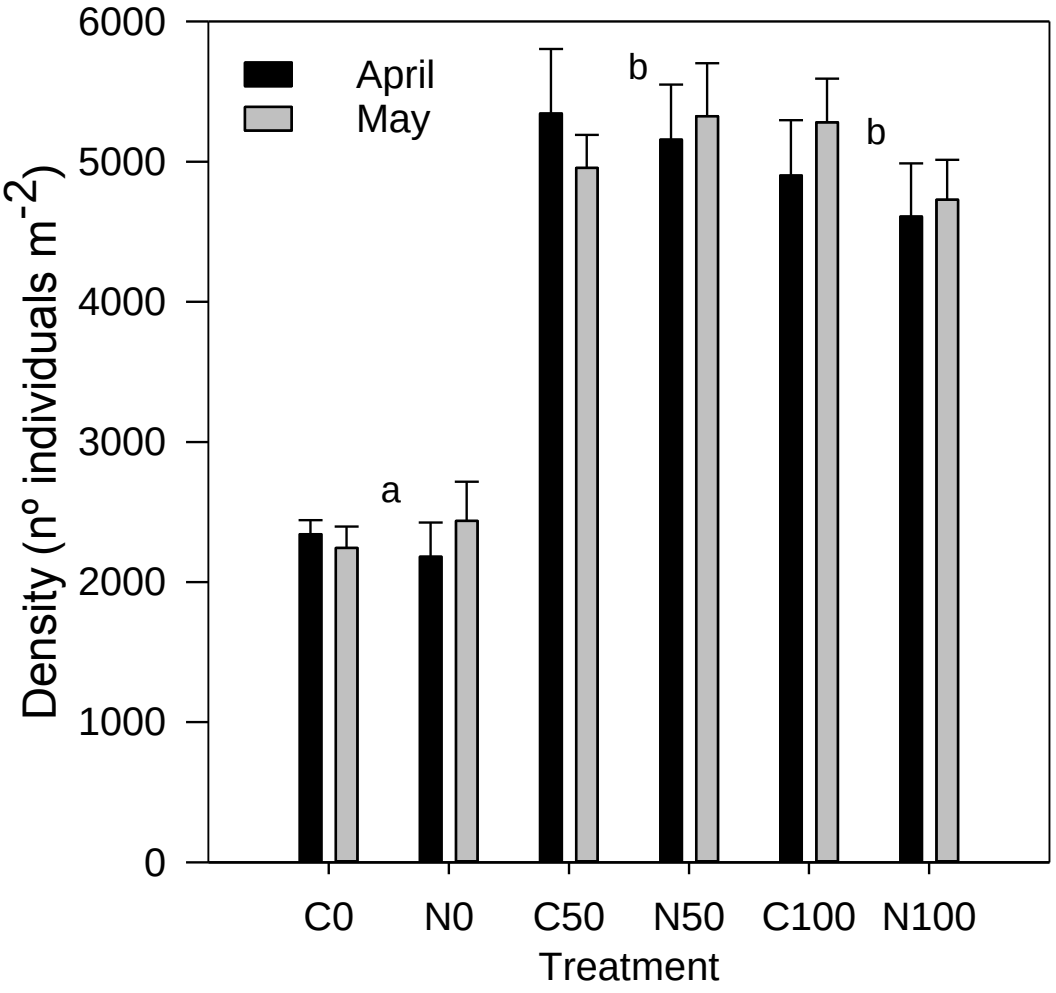

Fig. S4 Plant density, in drought and N addition treatments in April and May. Mean ± SE, *n* = 4. Different letters indicate significant differences between drought treatments and refer to both April and May (Holm-Sidak, $P < 0.05$). C - no added N (control), N - N addition and the number next to the letters represent the number of days of extended drought.

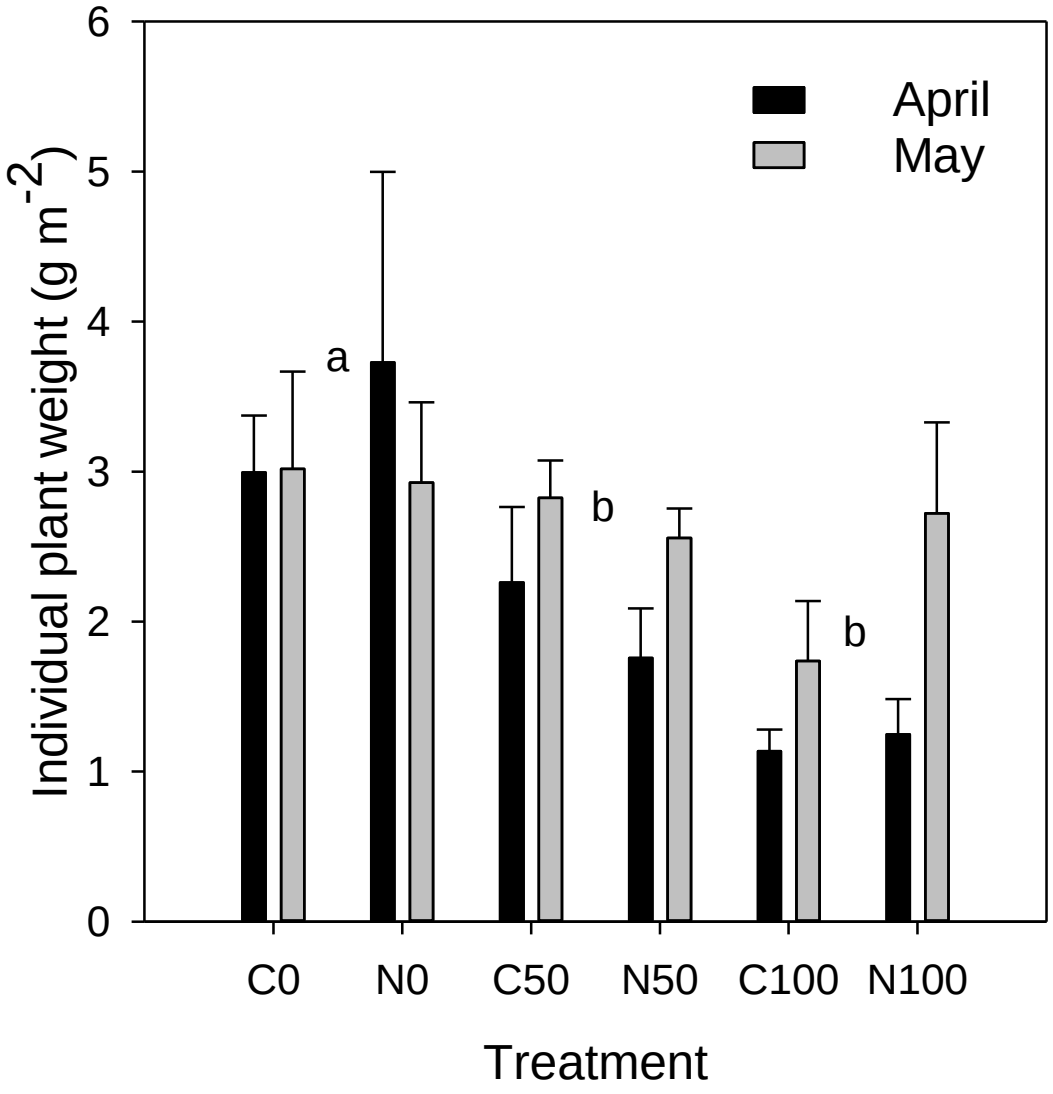

Fig.S5 Individual plant species weight, in drought and N addition treatments in April and May. Mean ± SE, *n* = 4. Different letters indicate significant differences between drought treatments and refer to both April and May (Holm-Sidak, $P < 0.05$). C - no added N (control), N - N addition and the number next to the letters represent the number of days of extended drought.

CHAPTER 3

Drought and nitrogen deposition interact to affect carbon fluxes in a Mediterranean grassland

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3 Drought and nitrogen deposition interact to affect carbon fluxes in a Mediterranean grassland

3.1 Abstract

After a long hot and dry summer, first rains in autumn trigger CO_2 and water fluxes in Mediterranean grasslands dominated by annual C3 species. Climate change scenarios project altered rainfall patterns, including a prolonged dry season into the autumn. Also nitrogen (N) deposition is increasing globally. How these global change drivers will interact to affect Mediterranean grassland CO_2, water fluxes and productivity is still unclear.

In a pot greenhouse experiment, we imposed three levels of extended drought in a factorial design: no drought, 50 days and 100 days of drought and two levels of N deposition: no N and N addition, to the seedbank of a Mediterranean grassland dominated by annual species.

Increased growing season length gave communities not exposed to drought an important development advantage. No drought translated into a higher net ecosystem exchange, higher gross primary productivity and aboveground biomass at the end of the growing season compared to the drought treatments. Communities subjected to 100 days drought had the lowest water use efficiency. N addition interacted with 100 days drought to attenuate the negative effects of drought on total CO_2 fluxes, however not affecting aboveground biomass or functional group composition. Both drought treatments affected negatively productivity of forbs the dominant functional group in our grassland. Our results anticipate important effects of interacting global change drivers on Mediterranean grassland functioning.

Keywords: aboveground biomass; drought; ecosystem respiration; evapotranspiration; gross primary productivity; Mediterranean grassland; net ecosystem exchange; nitrogen deposition

3.2 Introduction

Grasslands occupy approximately 40% of the earth surface playing an important role in the exchange of CO_2 and water with the atmosphere (Suyker and Verma, 2001; Aires et al., 2008a, b). In the Mediterranean Basin, grasslands alone or as understory of oak woodlands are biodiverse rich systems and important providers of ecosystem services, such as fodder production or CO_2 sequestration (Bugalho et al., 2011). However, droughts and increasing nitrogen (N) deposition, two important drivers of global change, are altering the structure and function of these grasslands (Zavaleta et al., 2003; Harpole et al., 2007; Pereira et al., 2007; DeMalach et al., 2017). Nonetheless, the magnitude of their effects, and in particular, their interactions remain largely unknown (Smith et al., 2016; Lei et al., 2016).

Mediterranean grasslands are dominated by annual C3 species, which typically germinate in autumn with the first major rain events (Espigares and Peco, 1995, Peco et al., 1998), have maximum growths in spring and senesce with the onset of the dry season (e.g. Xu and Baldocchi, 2004). These grasslands have a high inter-annual variability in productivity, CO_2 and water fluxes (Huxman et al., 2004 a,b; Pereira et al., 2007; Ma et al., 2007; Aires et al., 2008 a,b; Miranda et al., 2009; Jongen et al., 2011; Piayda et al., 2014) due mainly to the amount and seasonality of precipitation and occurrence of droughts, that also determine the length of the growing season (Huxman et al., 2004a; Ma et al., 2007; Jongen et al., 2011; Piayda et al., 2014). For instance, in a Mediterranean grassland in southern Portugal, during a dry year, total annual GPP (Gross Primary Productivity), ecosystem respiration and evapotranspiration were reduced by 58%, 55% and 34% respectively in comparison to a normal precipitation year (Aires et al., 2008a, b).

Adding to climatic changes, N inputs from fossil fuel combustion and fertilization have also globally increased (Peñuelas et al., 2013). N is one of the major limiting nutrients affecting primary productivity (e.g. Vitousek and Howard, 1991; Cleland and Harpole, 2010), which addition stimulates photosynthetic capacity and total leaf area and, thus positively affects GPP (Niu et al., 2010; Pérez-Priego et al., 2015a; Guo et al., 2016). Simultaneously, N addition may also have a positive effect on ecosystem respiration (Luo and Zhou, 2006) which can counter balance effects on net CO_2 ecosystem exchange (NEE) (Niu et al., 2010). Nonetheless, N effects on primary productivity depend strongly on water availability (Xia and Wan, 2008). Mediterranean grasslands are frequently co-limited by water and N (Hooper and Jonhson, 1999; Harpole et al., 2007), due to low precipitation and soil N

content. However, studies addressing interactions between drought and N addition and its effects on grassland CO_2 and water exchange are scarce (Lei et al., 2016, but see; Harpole et al., 2007; Niu et al., 2009; Guo et al., 2016). Particularly, there are few studies addressing the importance of the delay in autumn precipitation (autumn drought), that determines the start of the grassland growing season, and N availability on Mediterranean grassland productivity and CO_2 and water fluxes.

Climate change models forecast, for the Mediterranean region, more frequent and prolonged droughts (e.g. Kovats et al., 2014). In effect, the Mediterranean Basin is already undergoing more frequent extreme drought events (Paredes et al., 2006; Hoerling et al., 2012; Trigo et al., 2013). Also, N inputs, for the Mediterranean Basin, are forecasted to increase from 7 kg N ha^{-1} yr^{-1} in the mid-1990s to 12 kg N ha^{-1} yr^{-1} by 2050 (Phoenix et al., 2006). However, the potential ecological effects of N deposition on Mediterranean climate ecosystems have received little attention (Bobbink et al., 2010, but see Bonamoni et al., 2006; Dias et al., 2011 and Ochoa-Hueso and Stevens, 2015), mainly using realistic forecasted N loads (Cleland and Harpole, 2010, but see Ochoa-Hueso and Manrique, 2010). Most studies addressing the effect of N deposition on ecosystems have used high N addition (e.g 50-100 kg N ha^{-1} yr^{-1}) and few determined the interaction of N addition with drought (Smith et al., 2016).

The main goal of this study was to assess the effects of an extended summer/autumn drought and N deposition on the CO_2 and water fluxes of a Mediterranean grassland. To achieve this goal, we conducted a greenhouse pot experiment with grassland communities, where the duration of autumn drought and N addition (at the forecasted rate of 12 kg ha^{-1} yr^{-1}) were manipulated and chamber-based diurnal measurements of CO_2 and water fluxes performed.

We hypothesized that: (i) increasing drought duration would lead to a reduction in the length of the growing season, thereby decreasing total NEE and productivity of the grassland community; (ii) N addition would increase GPP resulting in a stronger net sink of CO_2 and thus attenuating negative drought effects; (iii) increasing drought and N addition would affect differently proportion of functional groups in the grassland community affecting CO_2 fluxes.

3.3 Materials and Methods

Study site and experimental setup

We conducted the experiment in a greenhouse situated at the School of Agriculture, University of Lisbon, Portugal, from 14th of October 2014 to 15th of May 2015. We used the top soil seed bank from a homogeneous semi-natural Mediterranean grassland, located in the Companhia das Lezírias estate, north-east from Lisbon (38^{o}49´45.13´´N, 8^{o}47´28.61´´W).

This grassland community was dominated by C3 annual species that germinate after first autumn rains (late September – October) and senesce at the onset of summer drought (mid-May - June). Dominant species included *Tolpis barbata* L., *Plantago bellardii* All. and *Tuberaria guttata* (L.) Fourr. (forbs), *Agrostis pourretii* Willd., *Avena barbata* Link and *Gaudinia fragilis* (L.) P. Beauv. (grasses), and *Ornithopus compressus* L., *Trifolium arvense* L. and *Trifolium campestre* Schreb (N-fixing species).

The climate is Mediterranean with hot and dry summers and mild winters. Mean annual temperature is 14.9 °C. Mean annual precipitation is 537 mm, approximately 84% of the precipitation falls between October and April. The soil is a well-drained deep Haplic Arenosol (IUSS, 2006) with a low water retention capacity and low total N content (0.18 mg N kg^{-1} dry soil).

We collected thirty-five quadrats of 20 cm x 50 cm containing the first 3 cm of soil surface of the grassland community, in mid-September, avoiding tree canopies (Luzuriaga et al., 2005). Samples were brought to the laboratory, thoroughly homogenized, sieved through a 3 mm mesh, and air-dried at room temperature for 48 hours.

In the greenhouse, forty-eight PVC pots (29.5 cm inner diameter and 24 cm height) were filled up with homogenized and roughly sieved dry soil from the grassland site. A thin layer containing 1000 cm^3 of the well mixed surface soil containing the seedbank of our grassland community was spread on each pot. These pots had a perforated base for efficient water drainage, lined with a gardening screen cloth to avoid soil loss and wrapped with a white plastic film to avoid excessive heating.

Each of the 48 pots was assigned randomly to one of six treatments, with eight replicates, considering a factorial design of three levels extended summer/autumn drought with (0, 50

and 100 days of extended drought) and two levels of N deposition (C-control or N-added). Specifically, treatments were, C0, C50, C100, N0, N50 and N100. For no extended drought, watering started on the 14th of October, for 50 days of extended drought watering started in the 3rd of December and for 100 days extended drought watering started on the 20th of January.

N was added in the form of ammonium nitrate (NH_4NO_3) at the rate of 12 kg N ha^{-1} y^{-1} which is the averaged projected rate of atmospheric N deposition in the Mediterranean Basin for 2050 (Phoenix et al., 2006). Irrigation with distilled water (no N addition) or with N fertilizer solution (N addition treatment), was performed twice a week in order to keep the soil moist throughout the whole experiment.

All pots were randomly distributed in the greenhouse and positions changed frequently. In addition, eight smaller control pots were filled with sterilized soil to test for background seed contamination. But no contamination was observed during the period of the experiment. The greenhouse had no supplemental light (attenuation was ca. 16%) or temperature control, following the natural weather variation.

Microclimate

Microclimatic conditions inside the greenhouse were continuously recorded. Relative humidity and air temperature were recorded every 30 min (VP-3 humidity temperature and vapour pressure sensor by an EM50 data logger, Decagon Devices, Pullman, USA). Daily integrated photosynthetic photon flux density (PPFD) was obtained from a meteorological station at the School of Agriculture. Volumetric soil water content (SWC) was continuously measured every 30 min using EC-5 soil moisture sensors (Decagon Devices, Pullman, USA) installed in three pots per treatment.

Ecosystem CO_2 and H_2O fluxes

Net grassland CO_2 exchange (NEE), ecosystem respiration (Reco) and evapotranspiration (ET) were measured using portable gas exchange chambers (39.5 cm × 39.5 cm × 54 cm) linked in closed dynamic circuit to an infrared gas analyzer (IRGA LI-840, Lincoln, NE, USA), measuring CO_2 and water vapour. Each measurement was no longer than three

minutes. Gas exchange chambers were made of 3 mm thick transparent plexiglass (> 95% light transmittance) to measure NEE and ET, and of opaque PVC, covered with an insulation layer of reflective aluminum foil to measure Reco. The chambers were tightly fitted to the pots and two small fans were fixed inside the chambers to continuously mix the air. A small pump circulated an air flow of 1 L min^{-1} through the sampling circuit and into an infrared gas analyzer (IRGA LI-840, Lincoln, NE, USA), which measures CO_2 and water vapour. Flux calculations and corrections for CO_2 water vapour dilution followed Pérez-Priego et al. (2015b) As Reco measurements were done approximately within one minute after NEE measurements, estimations of gross photosynthesis (GPP) were obtained by subtracting Reco from NEE. Ecosystem water use efficiency (eWUE) was estimated as -NEE/ET, following Scott et al. (2006). Diurnal time-courses (08h30, 12h00, and 16h00 hours) were conducted monthly (except in April, due to bad weather conditions), on clear sky days, over the course of the growing season on the 18th of December, 9th of January, 9th of February, 10th of March and 11th of May on three pots per treatment. Daily maximum CO_2 and H_2O exchanges were measured on the same dates and also on the 12th of November and 21st of January at noon. Air temperatures inside and outside the chambers were also registered. At each flux measurement date, leaf area index (LAI) was recorded as described below. Maximum CO_2 and water fluxes for each pot were used to estimate total Reco, NEE, GPP and ET at the end of the growing season. Our aim was to assess differences in the total CO_2 and water fluxes between treatments rather than the seasonal dynamics.

LAI

LAI in each pot was estimated with five intercalar PAR readings above and below vegetation, using the external PAR sensor of the ceptometer. PAR readings were taken close to solar noon, approximately every fortnight. LAI estimates were only possible when plant height exceeded PAR sensor height (≈ 7 cm). LAI in May was corrected for senescence based on aboveground biomass.

Aboveground biomass

Aboveground biomass was determined on four replicates per treatment on two sampling dates (15th of April and 15th of May) (n = 4, total of 24 pots on each sampling date). All plants in the pots were clipped at ground level, separated into functional groups (forbs, graminoids and legumes). Biomass was oven dried at 60°C for 48 hours prior to weighing.

Statistical analysis

Statistical analysis was performed using Sigmaplot 13.0 (Systac Software, Inc., SanJose, USA) and IBM SPSS Statistics 23.0 (IBM Corp. Armonk, NY, USA). Dependent variables were tested for homogeneity of variances, normality and sphericity (for repeated measure analysis) and transformed whenever necessary. Significance level was set to P = 0.05. A three-way ANOVA, with extended drought, N addition and month as factors, was used to analyze data on aboveground biomass and functional group proportion A two-way ANOVA, with extended drought and N addition as factors was used to analyze differences in LAI between treatments on each measurement date and effects of treatments on eWUEsum for the period between February and May. Maximum NEE, Reco and GPP (NEE_{max}, $Reco_{max}$ and GPP_{max}) were analyzed using a repeated measure ANOVA, with time, extended drought and N addition as factors. Tukey or Holm-Sidak pairwise multiple comparison procedures were applied to determine individual differences between means. A one-way ANCOVA was used to quantify the relationship between levels of extended drought and NEE_{sum}, $Reco_{sum}$, GPP_{sum} and ET_{sum}, controlling for N addition. NEE_{sum}, $Reco_{sum}$, GPP_{sum} and ET_{sum} corresponded to the sum of daily of gas exchange values for our seven measurement dates. Linear regressions were used to relate NEE_{max}, $Reco_{max}$, GPP_{max} and ET_{max} to LAI.

3.4 Results

Microclimate conditions

Average air temperature throughout the study was 15.4ºC, with October and May, being the hottest months with average temperature of 22.9ºC and 19.3ºC, respectively (Fig. 1). Maximum values in vapour pressure deficit (VPD) were also registered during those

months and in March and April (Fig. 1). January and February were the coldest months with daily average temperatures of 11.9ºC and 12.0ºC, respectively (Fig. 1). After day 10 in February average air temperature begun to increase steadily, followed by daily integrated photosynthetic photon flux density (PPFD) that reached maximum values during April and May (Fig.1).

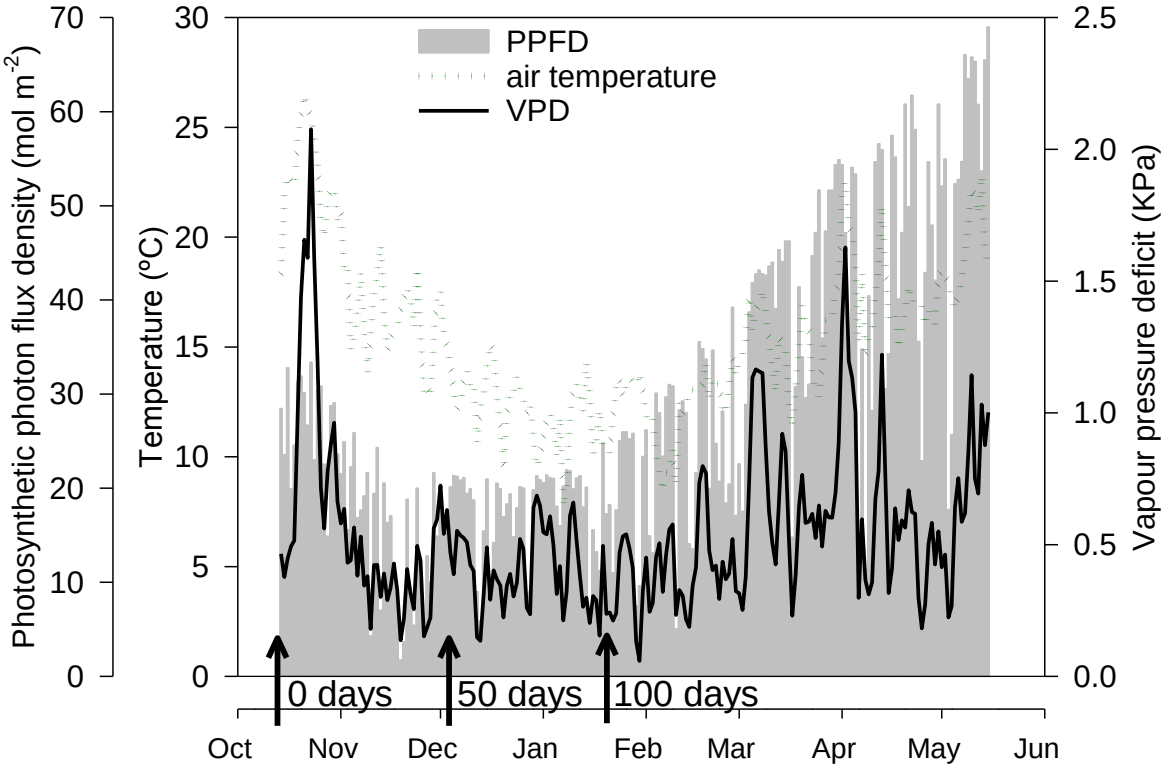

Fig.1 Daily integrated photosynthetic photon flux density (PPFD) (mol m^{-2} d^{-1}), daily average air temperature (ºC) and vapour pressure deficit (VPD) (kPa) over the course of the study. Arrows indicate date of first watering event for extended drought treatments with (N0, N50, N100) and without N addition (C0, C50, C100), with watering started on the 14th of October (0 days drought); on the 3rd of December (50 days extended drought); and 20th of January (100 days extended drought).

Soils were dry (SWC of 1.05% ± 0.37, mean ± SE) for the 50 and 100 days watering delay treatments, before first watering events. After watering, SWC averaged 22.37% ± 0.92,

which corresponded to 75% of the field capacity (≈30%), for all six treatments throughout the remaining growing season, with no substantial differences between all treatments.

Ecosystem CO_2 fluxes

Maximum CO_2 exchange fluxes were significantly affected by the extended autumn drought treatments (NEE_{max}: $F_{2,17} = 96.01$, $P < 0.001$, negative values indicate CO_2 uptake, while positive reveal release of CO_2; $Reco_{max}$: $F_{2,17} = 57.10$, $P < 0.001$ and GPP_{max}: $F_{2,17} = 109.75$, $P < 0.001$). However, direction and magnitude of responses depended on date of measurements (NEE_{max}: $F_{12,17} = 43.71$, $P < 0.001$, $Reco_{max}$: $F_{12,17} = 12.57$, $P < 0.001$ and GPP_{max}: $F_{12,17} = 49.74$, $P < 0.001$) (Fig. 2a, b and c). N addition did not have a significant effect on maximum CO_2 fluxes.

The no drought treatments (C0 and N0) had significantly higher NEE_{max} (i.e., more negative values) and GPP_{max} than both drought treatments until late January (Fig. 2b, c). C0 and N0 exhibited a relatively constant NEE_{max} and GPP_{max} throughout most of the growing season, with high NEE_{max} and GPP_{max} values in December and March (Fig. 2b, c). By February, NEE_{max} and GPP_{max} of 50 days drought (C50 and N50) increased, becoming similar to no drought and significantly higher than 100 days drought (C100 and N100) (Fig. 2b, c). In March, NEE_{max} and GPP_{max} of all treatments were high and similar, presenting no significant differences neither between drought and N treatments (Fig. 2b, c). Interestingly, from March to May, the NEE_{max} and GPP_{max} of the longest drought treatments increased, becoming significantly higher than no drought and 50 days drought treatments that decreased during that time period (Fig.2b, c).

Until February, $Reco_{max}$ of C0 and N0 was relatively stable and in general were significantly higher compared to the drought treatments (Fig. 2a). At beginning of January, $Reco_{max}$ of N50 peaked reaching similar values to C0 and N0 and was significantly higher than C50 on this measurement date ($P = 0.041$) (Fig. 2a). In May, $Reco_{max}$, of 100 days extended drought and N50 treatment were significantly higher than the no drought treatment (Fig. 2a).

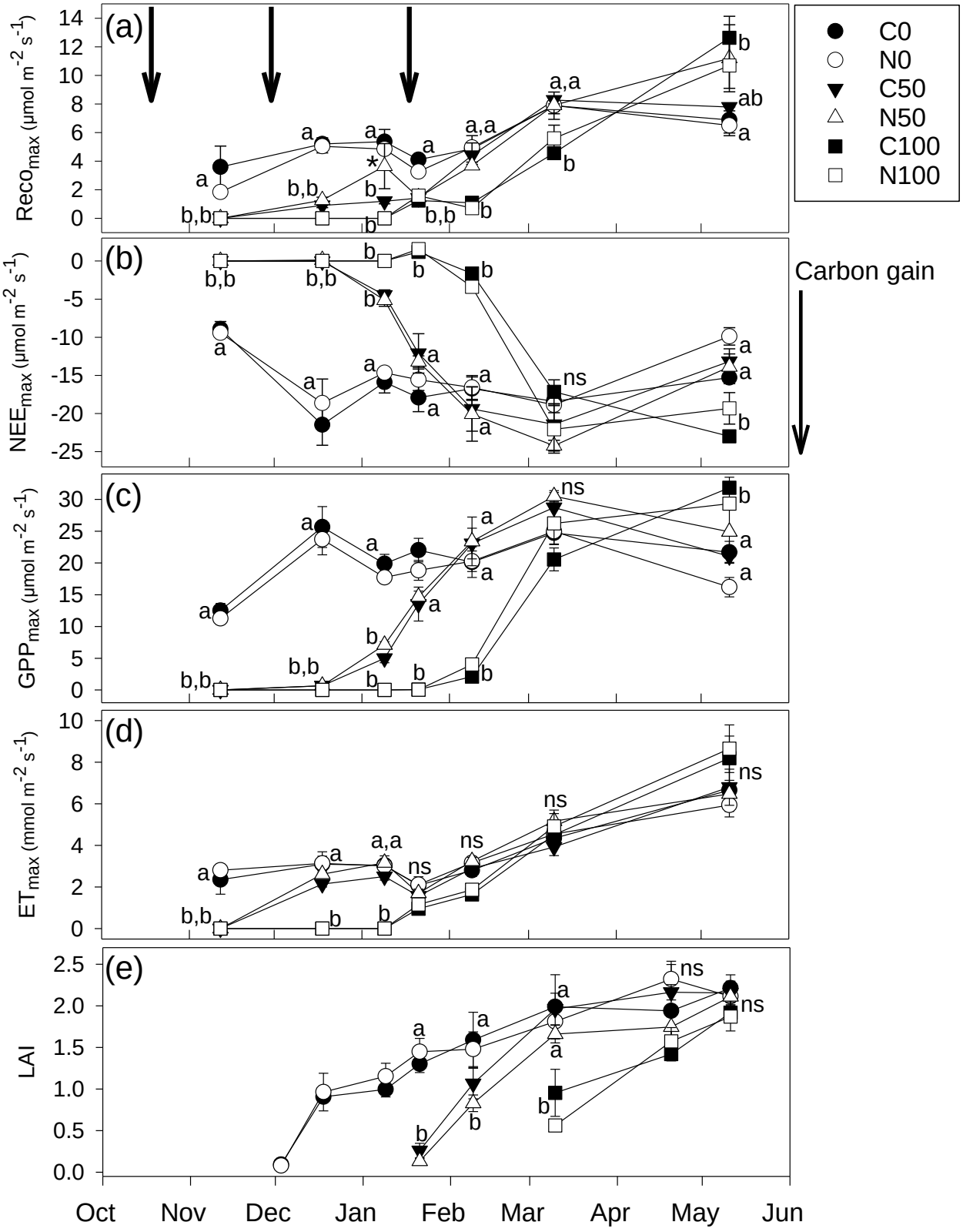

Fig. 2 Maximum CO_2 and H_2O daily fluxes and LAI throughout the growing season determined for all treatments: (a) $Reco_{max}$, (b) NEE_{max}, (c) GPP_{max}, (d) ET_{max} and (e) LAI. C - represents no added nitrogen (control), N - represents added nitrogen, the number next to the letters represent number

of days of drought. Arrows at the top indicate date of first watering event, 14th of October (no drought); 3rd of December (50 days drought) and 20th of January (100 days drought). Symbols show mean ± SE, n = 3. For each measurement date, different letters indicate significant differences (P < 0.05) between drought treatments; ns refers to no significant difference between drought treatments; asterisk (*) in $Reco_{max}$ in early January indicates a significant difference between C50 and N50.

Analysis of covariance (ANCOVA) indicated a significant negative effect of extended drought on the sum of daily NEE_{max}, $Reco_{max}$ and GPP_{max} (NEE_{sum}: $F_{2,17}$ = 83.73, P < 0.001, $Reco_{sum}$: $F_{2,17}$ = 26.08, P < 0.001 and GPP_{sum}: $F_{2,17}$ = 82.60, P < 0.001) (Fig. 3 a, b and c). Interestingly, N addition had a significant positive effect on $Reco_{sum}$ ($F_{1, 17}$ = 6.67, P = 0.024). Moreover, for all three CO_2 fluxes a significant interaction between drought and N was found (NEE_{sum}: $F_{2, 17}$ = 9.52, P = 0.003; $Reco_{sum}$: $F_{2, 17}$ = 5.10, P = 0.025 and GPP_{sum}: $F_{2, 17}$ = 11.15, P = 0.002) (Fig. 3 a, b and c). N addition had a significant positive effect on all three flux parameters above the 50 days of extended drought, i.e, in the 100 days drought treatment (NEE_{sum}: t = 4.27, P = 0.001; $Reco_{sum}$: t = 3.59, P = 0.004 and GPP_{sum}: t = 4.52, P < 0.001) (Fig. 3 a, b and c).

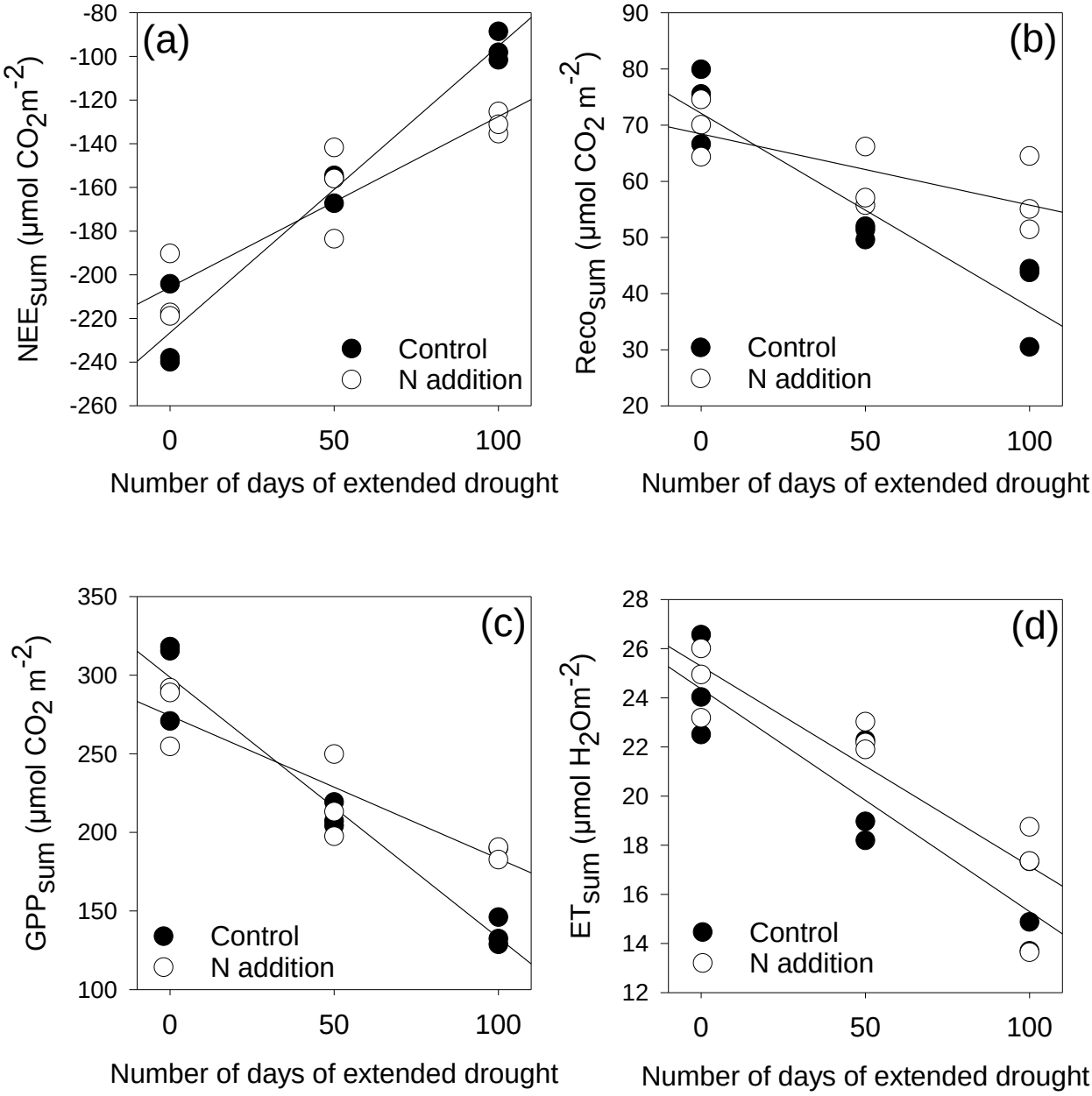

Fig. 3 NEE_{sum} (a); $Reco_{sum}$ (b); GPP_{sum} (c) and ET_{sum} (d), considering our seven measurement dates throughout the growing season, for each extended drought and N addition treatment. Symbols represent sum of NEE, Reco, GPP and ET per pot for the different treatments and lines represent regression lines.

Ecosystem water fluxes and water use efficiency

Extending the autumn drought significantly affected the seasonal patterns of ET_{max} ($F_{2,17}$ = 31.06, P < 0.001) with drought effect depending on date of measurement ($F_{12,17}$ = 11.72, P < 0.001) (Fig. 2d). However, N addition did not have a significant effect on ET_{max}. ET_{max} of

C50 and N50 treatments, 8 days after germination in December, were already similar to those of C0 and N0. By February, all three levels of drought and N treatments had similar ET_{max} and increased, reaching a maximum in May. In May, ET_{max} of 100 days drought treatment was almost significantly higher than no drought and the 50 days drought treatments (t = 2.55, P = 0.065 and t = 1.78, P = 0.095, respectively). The sum of daily ET (ET_{sum}) decreased significantly with drought extension ($F_{2,17}$ = 33.42, P < 0.001) (Fig. 3d), nonetheless the slopes of this relationship were similar with and without N addition.

The sum of $eWUE_{max}$, for the period between February and May, when germination had already occurred in all treatments, was significantly different between drought treatments ($F_{2,17}$ = 16.71, P < 0.001), with communities subjected to 100 days drought recording lowest $eWUE_{sum}$ (Fig. 4). N addition had no effect on $eWUE_{sum}$.

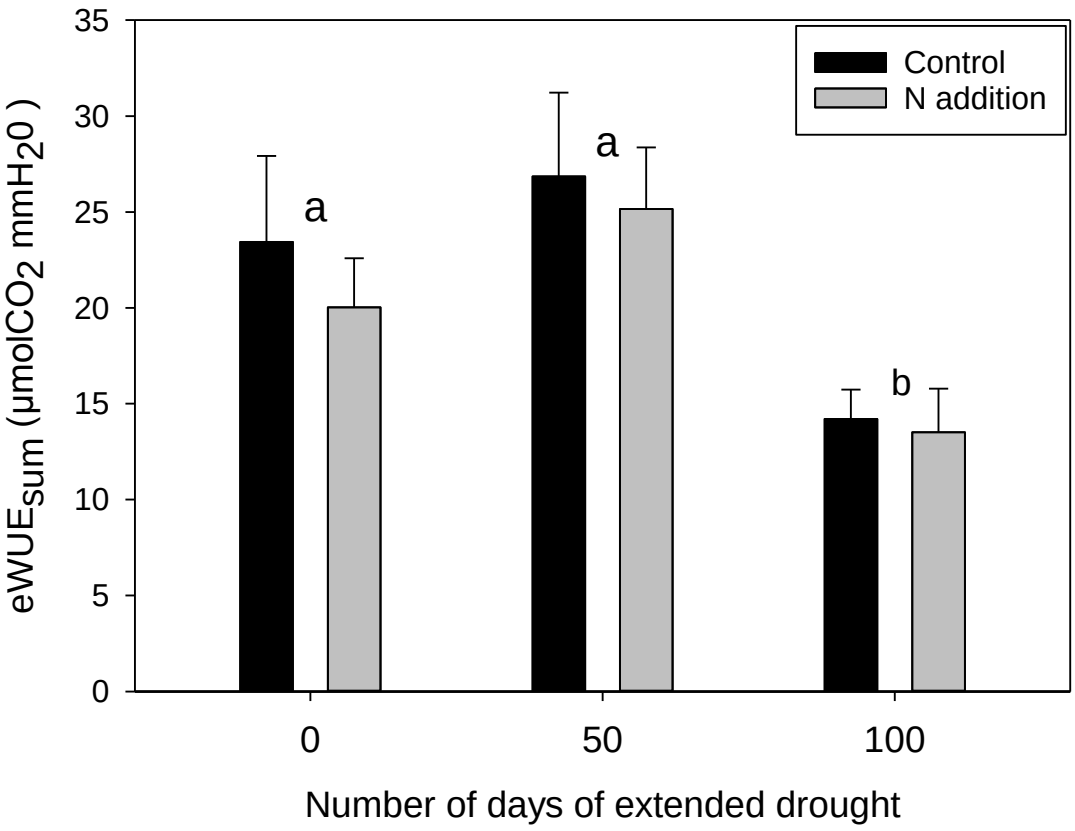

Fig. 4 Sum of ecosystem water use efficiency ($eWUE_{sum}$) considering our seven measurement dates throughout the growing season, for each extended drought and N addition treatment. Bars show mean sum of $eWUE_{sum}$ ± SE, *n = 3*. Different letters indicate significantly different means between drought treatments (Tuckey method, P < 0.05).

Development of LAI in relation to gas exchanges

LAI of C0 and N0 increased during winter reaching a LAI of 0.93 ± 0.06 (43% of its maximum LAI) in December. By March, C50 and N50 reached a similar LAI to that of the no drought treatment (Fig. 2e) and by April, all treatments had a similar LAI (Fig. 2e). For all LAI measurement dates there was no effect of N addition.

Changes in NEE_{max}, $Reco_{max}$, GPP_{max} and ET_{max} over the course of the growing season, were concurrent with changes in LAI (NEE_{max}: $F_{1,101}$ = 90.45, P < 0.001, R^2= 0.47; $Reco_{max}$: $F_{1,101}$ = 321.14, P < 0.001, R^2 = 0.76; GPP_{max}: $F_{1,101}$ = 151.12, P < 0.001, R^2 = 0.60; ET_{max}: $F_{1,101}$ = 213.13, P < 0.001, R^2 =0.68).

Community productivity and composition

Total aboveground biomass decreased significantly with increasing drought ($F_{2,47}$ = 57.61, P < 0.001). However, there was a significant interaction between drought and sampling month ($F_{2,47}$ = 13.56; P <0.001). Total aboveground biomass was not affected by N addition (Fig. 5). In April, aboveground biomass differed significantly between all drought treatments (Fig. 5), while in May, differences were only significant between no drought and 100 days drought (average 20.5% lower) (Fig. 5). Between April and May there was an overall strong productivity recovery for 50 and 100 days drought treatments (Fig. 5).

Regarding different functional group behavior, forbs was the most abundant functional group in all six treatments (78.85% ± 1.11, average across all treatments), followed by graminoids (15.42% ± 0.86) and legumes (5.74% ± 0.68). Relative abundance of forbs decreased with drought treatments ($F_{2,47}$ = 0.01, P < 0.001), whereas relative abundance of graminoids and legumes increased with drought ($F_{2,\ 47}$ = 6.45, P = 0.004 and $F_{2,47}$ = 80.74, P < 0.001, respectively). Relative abundance of graminoids was favored by the 50 days drought. Functional groups were unresponsive to N addition.

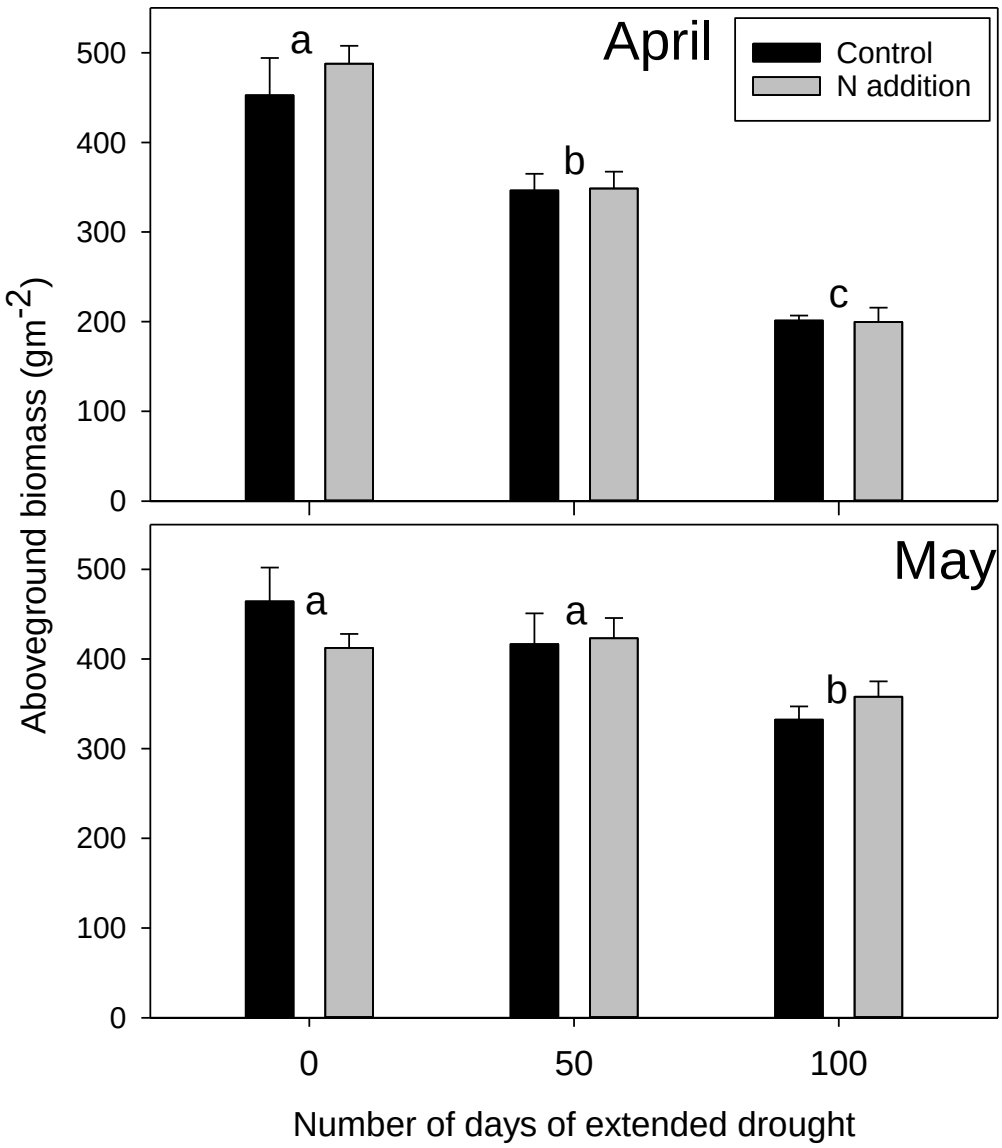

Fig. 5 Aboveground biomass in April and in May, for extended drought and N addition treatments. Bars show mean ± SE, *n* = 4. Different letters indicate significantly different means between drought treatments (Tuckey method, $P < 0.05$).

3.5 Discussion

Our results highlight the negative effects of extended autumn droughts on CO_2 and water exchange of Mediterranean grassland communities and of a positive effect of N addition on net CO_2 exchange in the severe extended drought (100 days), stressing the importance of interacting global change drivers on Mediterranean grasslands.

In Mediterranean grasslands, the length of the growing season plays an important role in ecosystem CO_2 uptake (Ma et al., 2007; Jongen et al., 2011; Piayda et al., 2014), that is linked to the timing of first rain events in the early autumn (Xu and Baldocchi, 2004; Ma et al., 2007; Aires et al., 2008a). For example, in a Mediterranean grassland in California, earlier autumn rains increased winter GPP in ~90 g CO_2 m^{-2} due to an earlier start of grass germination, even though total precipitation was low (Xu and Baldocchi, 2004). Also, Piayda et al. (2014) reported that GPP of the herbaceous understory of a savannah woodland in Portugal was reduced by 53% due to one-month delay of first autumn rains and a reduction in winter precipitation. As a consequence, and in agreement with our results, these authors observed a substantial delay in seed germination, in leaf area growth and a later start of CO_2 sequestration, with significant negative consequences for the annual CO_2 balance. Indeed, favorable growing conditions at the beginning of our experiment gave C0 and N0 communities an important development advantage in LAI. Increased leaf area stimulated by mild temperatures and water availability (Xu and Baldocchi, 2004), allowed a rapid dominance of GPP over Reco, reinforcing the importance of autumn growth for total CO_2 fluxes. An early grassland cover development has not only positive effects on total CO_2 fluxes but may also additionally have strong impacts on soil water relations, such as reduction of soil evaporation and increased rain infiltration, especially under strong rain events (e.g. Dubbert et al., 2014a) that are typical of Mediterranean climates.

By extending autumn drought, the length of the growing season was reduced and also the environmental conditions, to which our communities were exposed, from germination to senescence, were altered. For instance, accumulated temperatures above a certain growing threshold (i.e., growing-degree days index; GDD), which determine plant growth and phenology (e.g. Peñuelas and Fillella, 2001), decreased substantially with increasing drought (Nogueira et al., 2017). Total PPFD, over the length of the growing season, decreased 16% and 31% for 50 days and 100 days, comparing with no drought treatments, which most probably affected GPP (Aires et al., 2008a).

Variations in GPP, NEE, and ET throughout the growing season were highly correlated with seasonal differences in LAI, as the potential to intercept light for photosynthesis or for water vapour exchanges are limited by leaf area and physiological capacity (Aires et al; 2008 a,b; Jongen et al., 2011). Although, LAI has a large effect on ecosystem CO_2 exchange, leaf-level photosynthetic capacity also varies during the season (Huxman et al.,

2004c), which may explain the spring decrease in GPP in no drought treatments, compared to the younger communities in the 100 days drought treatment. Higher proportion of graminoids and legumes with a higher specific leaf area (higher metabolic rates) compared to rosette species (e.g. forbs), for instance, (Westoby, 2002; Ansquer et al., 2009) may further have caused the faster response in GPP in the 50 and 100 days drought treatments, which translated into an important aboveground biomass increment from April to May, in the two more prolonged drought treatments. In fact, the functional group composition of these grasslands is highly dynamic and responsive to environmental conditions and, specifically to drought (e.g. Espigares and Peco, 1995, Peco et al., 1998; Dubbert et al. 2014b; Nogueira et al., 2017).

Extending autumn drought, and thus postponing germination latter into the growing season has, however, the drawback that phenologically younger communities with high rates of CO_2 accumulation and transpiration will grow when evaporative demand is greatest (Huxman et al., 2004c; Aires et al., 2008b). The decrease in eWUE with increased extended drought combined with the observed decrease in total GPP may reduce resilience in these communities under the predicted scenario of a prolonged dry season or more frequent dry spells (Jongen et al., 2014).

In Mediterranean ecosystems, the increase in soil moisture, with first autumn rains, leads to a burst of decomposition, mineralization and release of inorganic N and CO_2 (Huxman et al., 2004c; Jarvis et al., 2007; Unger et al., 2010, 2012). Longer the previous drought period, the greater are the decomposition and mineralization rates on rewetting (Jarvis et al., 2007; Unger et al., 2010). In annual dominated grasslands, this N input from mineralization with first autumn rains is most probably lost through leaching as annuals have not yet germinated and there are no other plant life forms (e.g. perennials) that can use the available N (Joffre et al., 1990). Subsequent ecosystem CO_2 uptake will depend on ensuing precipitation, germination and photosynthesis, as well as on the magnitude of Reco, through its responses to temperature and soil water (e.g. Jongen et al., 2011).

GPP increased more along the growing season than did Reco as GPP follows the seasonal dynamics of PAR and air temperature (Gilmanov et al., 2007). Slower increment of Reco throughout the growing season can be explained by the fact that Reco is dependent on the availability of substrates, such as root exudates, supplied by photosynthesis (Reichstein et al., 2003; Aires et al., 2008a ;Li et al., 2017) that increase

with LAI and GPP (Reichstein et al., 2003). However, time lags in the response of Reco to GPP have been reported for herbaceous species (Fitter et al., 1998) and thus the response of Reco to photosynthesis can be masked by more sensitive drivers such as temperature and moisture (Luo and Zhou, 2006). Differences in the proportion of functional groups between treatments may also have influenced Reco. Legume species, for instance, have a positive effect on soil respiration (Tjoelker et al., 2005; Li et al., 2012) which might have contributed to similar Reco values in February between controls and 50 days drought, despite the latter having significantly lower LAI.

As our community was N limited (Spehn et al., 2002) we hypothesized that the positive relationship between N addition, photosynthetic rate and LAI (Niu et al., 2009, 2010) would attenuate the negative effects of drought on GPP. Also, expected stimulation of leaf photosynthesis would provide more substrate for soil and plant respiration, leading to an increase in Reco under N addition (Niu et al., 2010), although contradictory or null results have also been shown (e.g. Bubier et al., 2007; Janssens et al., 2010). In our study, there was a positive N effect on total NEE (more negative values) under a severe drought, with N addition attenuating the negative effect of increasing drought on both Reco and GPP. The positive effect of N addition on NEE suggests that under the occurrence of autumn droughts, as predicted by climate models, N deposition may increase CO_2 sequestration in Mediterranean grasslands. Also, our results suggest a synchronicity of microclimatic conditions, N availability and plant life-stage that might have stimulated community response in the severe drought treatment (Niu et al., 2005; Tulloss and Cadenasso, 2016). Indeed, in the 100 days drought treatment, watering begun when air temperatures were low, and thus N mineralization rates where probably lower (Jarvis et al., 2007). This could partly explain why total CO_2 sequestration was lower in C100 compared to N100, as N addition must have gradually stimulated differences in CO_2 fluxes. Also, and more importantly N uptake by plants has usually a peak in early spring (Eviner and Firestone, 2007). The younger communities of the 100 days treatments, that had a high development increment during that period, must have beneficiated from the supply of N.

We found that late arrival of autumn rains affects the establishment of herbaceous species, with consequences for community composition and biomass production (Espigares and Peco, 1995; Miranda et al., 2009). Accordingly, we observed differences in relative abundance of functional groups and a significant negative effect on aboveground biomass. Cooler temperatures in December and January when first watering of the 50 and

100 days drought treatments occurred, favored the establishment of legumes species, whereas large biomass forb species (e.g. some rosette species) were favored by early autumn rains (Espigares and Peco, 1993; Del Pozo and Aronson, 1999; Nogueira et al., 2017).

Higher total summed GPP with N addition did not translate into increased aboveground biomass. A lack of productivity response to N addition, within the predicted N deposition values for the Mediterranean Basin (10 and 20 kg N $ha^{-1}year^{-1}$), was also found by Ochoa-Hueso and Stevens (2015), in a semiarid shrubland in central Spain. The authors observed biomass species-specific responses and a significant change in plant community composition, with responses to N occurring approximately 2.5 years after N addition and controlled by precipitation and P availability. In our study, despite changes in functional group composition with drought, forbs still represented ca. 79% of our grassland community. Forbs, most probably included species with N conserving strategies such as low N tissue concentration and low relative growth rates (low specific leaf area) (Craine et al., 2001; Ansquer et al., 2009) which might help to explain the lack of response to increased N availability (Cleland and Harpole, 2010). Responses to N addition must be considered in the context of multiple interacting factors (Bobbink et al., 2010; Cleland and Harpole, 2010). The increase in growth might be limited when soil chemical properties (e.g. pH), other nutrients, such as P and base cations, become more limiting than N (e.g. Bobbink et al., 1998). In fact, we have observed in the field, that our grassland is P limited (personal observation). Additionally, life-stage responses of individual species to N addition may also mask overall grassland responses (Tulloss and Cadenasso, 2016).

3.6 Conclusions

Our results showed that an extended autumn drought had a significant negative impact on the Mediterranean grassland community leading to changes in community composition, and a decrease in total CO_2 and water fluxes and aboveground productivity due to a shortening of the effective growing season. We found that N addition attenuated the negative effects of a severe autumn drought on ecosystem CO_2 fluxes. This is especially relevant in Mediterranean climates with marked precipitation seasonality where droughts significantly reduce CO_2 sequestration and where prolonged summer droughts are projected to occur. Differences in aboveground biomass observed in early spring, between

drought treatments, anticipate that resilience of Mediterranean grasslands might be negatively affected by the combination of an extended autumn drought and the forecasted decrease in spring precipitation. Our results emphasize the importance of interacting global change drivers on the functioning and resilience of Mediterranean grassland ecosystems and point out to the complexity of responses of these diverse ecosystems to these drivers.

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CHAPTER 4

Nutrient addition in a Mediterranean grassland decreases species diversity, increases productivity but does not affect stability

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4 Nutrient addition in a Mediterranean grassland decreases species diversity, increases productivity but does not affect stability

4.1 Abstract

Increased nutrient inputs and climate change are affecting ecosystems worldwide. However, there is a dearth of knowledge on how the interacting effects of multiple nutrient inputs and climatic variability may affect ecosystem functioning including grassland species diversity, productivity or resilience to disturbances. This is particularly important in the Mediterranean Basin, a hotspot of climate change, where the frequency of autumn and spring droughts is projected to increase.

We conducted a 5-year nutrient addition experiment in an annual Mediterranean grassland. We added nitrogen, phosphorus and potassium in a factorial way to grassland communities to establish three treatments of one, two and three added nutrients. We also maintained a control without addition of nutrients. We examined how increased number of nutrients and inter-annual climatic variability affected grassland diversity, productivity and resilience to a disturbance event. Resilience was decomposed in resistance and recovery after a combined severe drought and heavy grazing disturbance event that occurred in the third year of the experiment.

Grassland productivity was co-limited by multiple nutrients and precipitation. Nutrient addition decreased species richness and interacted with climatic variability to alter functional group composition. Grassland resilience to a grazing and drought disturbance event was not affected by nutrients availability through a counterbalance between resistance and recovery. Grassland resistance to the disturbance decreased in nutrient enriched communities probably due to lower species richness, but recovery increased led by the dominance of competitive graminoid species.

This study shows that mechanisms underlying ecological functioning and resilience of Mediterranean grasslands depend on interactions of multiple nutrient addition and precipitation variability. Understanding these mechanisms is crucial to anticipate potential effects of global changes on Mediterranean grasslands.

Keywords: functional groups, Mediterranean grassland, multiple nutrients, productivity, recovery, resilience, resistance, richness, stability.

4.2 Introduction

Grasslands cover approximately 40% of the terrestrial surface, harbor high biodiversity, and generate essential ecosystem services (Myers et al., 2000; Hector and Bagchi, 2007; Bugalho and Abreu, 2008). When temperature is not limiting, grassland productivity depends strongly on annual precipitation (Knapp and Smith 2001; Huxman et al., 2004). Mediterranean grasslands, in particular, are very responsive to changes in water availability and precipitation (Huxman et al., 2004; Knapp et al., 2015). Still, nutrient availability is also a strong determinant of grassland productivity (Chapin, 1980; Gough et al., 2000; LeBauer and Treseder, 2008; Fay et al., 2015; La Pierre et al., 2016).

Mediterranean grasslands are adapted to high inter- and intra-annual precipitation variability (Peco et al., 1998; Espigares and Peco, 1993) and characterized by a rich species pool of mainly annual species that survive the unfavorable hot and dry season as seeds (Pitt and Heady, 1978, Díaz-Villa et al., 2003). Mediterranean regions are hotspots of climate change (Giorgi, 2006), where increased frequency and severity of spring and summer droughts (Miranda et al., 2002, Kovats et al., 2014) are expected to strongly affect Mediterranean grassland productivity and diversity (Peco et al., 1998; Zavaleta et al., 2003; Miranda et al., 2009, 2011).

Increased widespread use of fertilizers, specifically nitrogen (N) and phosphorus (P), and inputs of reactive N from fossil fuel combustion are other global change drivers increasing nutrient availability in the biosphere (Peñuelas et al., 2013). Most studies on natural and semi-natural grasslands have mainly focused on the effects of a single nutrient, mainly N, on grassland productivity and diversity (e.g. Gough et al., 2000; Stevens et al., 2010; Isbell et al., 2013). Studies involving other nutrients, such as P and potassium (K), although common in agricultural systems, are still rare in natural and semi-natural grasslands, (Kayser and Isselstein, 2005; Harpole et al., 2011; Ceulemans et al., 2014; Fay et al., 2015). Nutrient addition often stimulates grassland productivity but decreases plant diversity (e.g. Gough et al., 2000; Clark et al., 2007; Harpole et al., 2016), while nutrient limitation can increase niche dimensionality enabling the co-existence of species differing in nutrient requirements and competition ability (Harpole and Tilman, 2007; Cleland and

Harpole, 2010; Harpole et al., 2016). Adding increased number of nutrients usually induces a general loss of diversity through competitive exclusion of the poorest competitors (Harpole and Tilman, 2007; Harpole et al., 2016). Water availability, however, may also affect nutrient co-limitation (Hooper and Jonhson, 1999; Harpole et al., 2007; Cleland and Harpole, 2010; DeMalach et al., 2017) and ultimately grassland functioning and resilience to disturbances (Zavaleta et al., 2003; Dukes et al., 2005; Harpole et al., 2007). This is particularly relevant for Mediterranean grasslands likely to be affected by extreme drought events that may combine with other disturbances such as grazing. Grazing can be seen as a key structural disturbance likely modifying grassland species composition, richness, plant functional traits and productivity (Altesor et al., 2005; Pérez-Camacho et al., 2012; Rota et al., 2017). However, effects of grazing on grasslands can depend on precipitation variability and amount (Pérez-Camacho et al., 2012; Carmona et al., 2015; Rota et al., 2017).

Therefore, understanding interactions between nutrient addition and precipitation variability and how these interactions may condition Mediterranean grassland responses to drought and grazing, is critical to anticipate effects of global changes on grassland functioning and resilience.

Here, we conducted a 5-year (2013-2017) study in a Mediterranean grassland, to determine how increased number of nutrients interacted with climate variability to affect grassland diversity and productivity. We took advantage of a disturbance event (heavy grazing and drought) that occurred in 2015 to further study the effect of nutrient addition on grassland resilience.

We hypothesized that increased nutrient addition will: (1) combine with increased precipitation negatively affecting grassland species richness but increasing grassland productivity; (2) homogenize grassland resilience to the combined drought and heavy grazing disturbance through dampening of resistance but increased productivity recovery.

4.3 Materials and Methods

Site description

Our study was conducted in a semi-natural Mediterranean grassland, located north-east of Lisbon, Portugal (38^{o}49´45.13´´N, 8^{o}47´28.61´´W). The grassland community, occasionally grazed by cattle until 2012, was dominantly composed of annual species, which emerge after first autumn rains and senesce and set seed in late spring. Dominant forbs are *Tolpis barbata* L., *Plantago bellardii* All. and *Tuberaria guttata* (L.) Fourr., grasses are *Agrostis pourretii* Willd., *Avena barbata* Link and *Gaudinia fragilis* (L.) P.Beauv., and legumes are *Ornithopus compressus* L., *Trifolium arvense* L. and *Trifolium campestre* Schreb.

The climate is Mediterranean, with mild, wet winters and hot, dry summers. Late Spring and Summer droughts typically extend from May through October and winter rains from November through April. Long-term (1961–1990) mean annual precipitation is 709 mm. Mean long-term annual temperature is 15.9 °C, ranging from 10 ºC in January to 22.5 ºC in August (INMG, 1991). Site topography is flat. The soil is a well-drained deep Haplic Arenosol (IUSS, 2006) with a low water retention capacity and N content (0.12%).

Experimental design

Plots of 5 m x 5 m, separated by 1-m walkways, were established in a randomized three block design. Addition of N, P and K plus micronutrients, was made in a factorial way, totaling eight different nutrient additions per block, including controls (no added nutrients). To establish a nutrient addition gradient (e.g. Harpole et al., 2016) we considered 4 treatments that varied in the number of added macronutrients (0, 1, 2 and 3). Thus, controls with no added nutrients are termed as 0; for 1 added macronutrient, we grouped plots with single macronutrient additions of N, P or K; 2 added macronutrients treatment were additions of NP, NK or PK, and 3 added macronutrients treatment were plots fertilized with NPK. One and two added macronutrients had each a total of 9 replicates and control and three added macronutrients had each a total of 6 replicates.

Hereafter, we use the term ‘number of added nutrients’ (0, 1, 2 or 3) to represent the number of potentially limiting elemental nutrients added.

Nutrient treatments were applied every fall, since 2012 until 2017. N was added from 2012 to 2016 as slow-release urea (60-90 days) at a rate of 10 g N m^{-2} yr^{-1}, switching in 2017 to two separate additions of urea (three months apart to mimic slow release) due to restricted availability of timed-release urea. P was added as triple-super phosphate and K as potassium sulfate, both at a rate of 10 g m^{-2} yr^{-1}. In the first year, a micronutrient mix (6% Ca, 3% Mg, 12% S, 0.1% B, 1% Cu, 17% Fe, 2.5% Mn, 0.05% Mo, and 1% Zn) was applied once (100 g m^{-2}) into K plots. We included the addition of K plus micronutrients as a single macronutrient although the addition of micronutrients in 2012 could have affected the outcome of K addition. The experiment is included in the Nutrient Network (www.nutnet.org; Borer et al., 2017).

The site was fenced in 2012 to avoid cattle grazing and trampling. At the end of each growing season and after seed production and dispersal, all standing vegetation in the fenced experimental area was cut with a brush cutter to approximately 5 cm aboveground, and removed, leaving only short stubbles and roots.

Air temperature and relative humidity (VP-3 humidity and temperature sensor, Decagon Devices, Pullman, USA), were recorded and logged every 30 min (EM50 data logger, Decagon Devices, Pullman, USA). Precipitation was also recorded (tipping bucket RG2, Delta-T Devices, Cambridge, UK) by connection to a logger (CR1000 data logger, Campbell Scientific, Logan, USA). Due to the Mediterranean climate of the region, we considered the annual grassland growing season, starting September 1 and ending May 7.

Species cover and richness

Species cover (%) of each plant species was visually estimated, to the nearest 1%, annually, at peak vegetation growth, in a permanently marked $1m^2$ quadrat in each plot. Species richness was computed every year as the number of plant species per plot.

Aboveground biomass

Aboveground plant biomass was used as a proxy of grassland productivity. Productivity was estimated destructively, every year, by clipping all individual plants rooted within two 10 cm x 100 cm strips per plot. The locations of the clipping strips were moved within each

plot every year to prevent clipping effects. In the laboratory, biomass was divided into functional groups (forbs, graminoids, and legumes). Biomass samples were dried to constant mass at 60 °C prior to weighing.

Functional group (graminoids, forbs and legumes) proportions were determined for each plot, as the productivity ratio of the biomass of a given functional group and the total biomass of that plot. Leaf area index (LAI) was estimated in each plot using a ceptometer (AccuPAR model LP-80, Decagon Devices, Pullman, USA). Photosinthetical Active Radiation (PAR) readings were taken close to solar noon, approximately every fortnight during active vegetation growth.

Climatic variability

We used the standardized precipitation evapotranspiration index (SPEI), which computes precipitation minus potential evapotranspiration accumulated over a given time-scale (Vicente-Serrano et al. 2010), to characterize the variable climatological conditions throughout the study. We defined, for each year: a grassland full growing season SPEI which integrated the period from September to April, and three different growing periods: fall SPEI (September, October and November), winter SPEI (December and January) and spring SPEI (February, March and April).

Resistance, recovery, resilience and stability

The dry winter and spring of 2015 (Table 1) combined with a heavy defoliation across the study area, resulting from an unscheduled cattle grazing in February, gave us the opportunity to evaluate grassland resistance, recovery, resilience and stability to a combined drought/grazing disturbance. We calculated resistance, recovery and resilience for total productivity following van Ruijven and Berendse (2010). Resistance was calculated as the difference in log productivity in the disturbance year of 2015 and before the disturbance in 2014. We estimated recovery as the difference in log productivity in 2016 the year post-disturbance and in the year 2015 of the disturbance. Resilience was calculated as the ratio of productivity in 2016, the post-disturbance year and in the year 2014, previous to the disturbance.

We used the coefficient of variation (CV= $\mathrm{standard\,deviation}/\mathrm{mean}$) based on five-year total productivity, as an additional stability metric (Scheffer et al., 2015).

Statistical analysis

We used a two-way repeated measure ANOVA with number of added nutrients and year as fixed factors and grassland diversity, productivity and functional group proportion as response variables to analyze grassland responses to nutrient addition across years. CV, resistance, recovery and resilience were analyzed using a one-way ANOVA, with number of added nutrients as the main factor. When a significant difference was found for a main factor, a Holm-Sidak pairwise multiple comparison procedure was applied to determine individual differences between means. Linear regressions were used to relate species richness, productivity and functional group proportion, to number of added nutrients and to relate species richness to resistance. To determine the relationship between N, P and K and functional group proportion, we used a linear regression with N, P and K as binary independent variables.

To explain productivity and functional group proportions among years we conducted a complete stepwise multiple linear regression with number of added nutrients, previous year productivity, number of years of nutrient addition, grazing, annual and seasonal SPEI as explanatory variables. We analyzed separately two sets of models considering fall, spring and winter SPEIs and the full growing season SPEI. To determine best predictors of functional group proportion, groups were examined separately, as forbs, graminoids and legumes are not independent from each other. To explain functional group proportion variability among years, we used the functional group proportion of the previous year (e.g. for legume variability we used the legume proportion of the previous year). Factors that were collinear were removed from the model in a stepwise way. When factors were collinear we tested each factor at a time. We used Akaike information criterion (AIC) to determine the best model. Dependent variables were tested for homogeneity of variances and normality and transformed when necessary. Level of significance was set to $P = 0.05$. Statistical analysis was performed with Sigmaplot 13.0 (Systac Software, Inc., San Jose, USA) and by IBM SPSS Statistics v. 23 (IBM, Chicago, IL, USA).

4.4 Results

Climate

During the study period, 2015 was the driest growing season, with a very dry winter and spring (65% and 70% below long-term precipitation mean, respectively), and a wet fall, resulting in a high precipitation variability (CV = 1.13) (Table 1). In 2016 and 2017, the growing season (fall, winter and spring) precipitation was, on average, 22% lower than the long-term mean. The year 2017 was the second driest, with a drier spring, particularly in April, with 28 consecutive days without rain. Also, spring temperatures were 2 ºC above the long-term mean (Table 1). These precipitation and temperature anomalies were clearly translated into the negative SPEI of the grassland growth season (Table 1).

Table 1 Total precipitation and mean air temperature for the growing seasons of 2013 to 2017 and for the long-term average (1961-1990) in: fall (1 September – 30 November), winter (1 December – 31 January), spring (1 February – 7 May (biomass harvest)); full growing season: includes fall, winter and spring. CV of precipitation for each year and for long term average is presented. SPEI was determined for fall, winter, spring and full growing season.

	2013	**2014**	**2015**	**2016**	**2017**	**1961-90 (long term average)**
Total precipitation (mm)						
Fall	268	232	462	156	150	200
Winter	124	211	73	148	148	207
Spring	278	221	67	171	161	220
Full growing season	670	664	602	473	460	627
CV	0.39	0.05	1.13	0.08	0.05	0.05
Average air temperature (ºC)						
Fall	16.4	16.4	18.2	17.2	17.6	17.3
Winter	10.8	9.9	8.8	12.5	9.7	10.2
Spring	12.4	12.9	13.4	13.1	14.5	12.4
Full growing season	13.2	13.0	13.5	14.3	13.9	13.3
SPEI						
Fall	0.92	-0.32	1.09	-0.22	-0.16	
Winter	0.28	0.12	-0.63	-0.29	-0.36	
Spring	0.79	0.74	-1.21	0.43	-0.74	
Full growing season	0.97	0.06	-0.37	-0.19	-0.79	

Diversity

Species richness decreased significantly with number of added nutrients ($F_{3,149} = 6.51$, $P = 0.002$; linear regression: $F_{1,119} = 20.65$, $P < 0.001$) and varied significantly across years ($F_{4,149} = 35.50$, $P < 0.001$), but there were no interactions between added nutrients and year ($F_{12,149} = 0.60$, $P = 0.838$) (Fig. 1). On average, species richness was lower in the three nutrients treatment, than in controls ($t = 3.76$, $P = 0.004$) and one nutrient treatments ($t = 3.91$, $P = 0.004$) (Fig. 1). Species richness declined significantly in 2017 compared to previous years (2013: $t = 8.76$, $P < 0.001$; 2014: $t = 11.25$, $P < 0.001$; 2015: $t = 5.51$, $P < 0.001$ and 2016: $t = 6.86$, $P < 0.001$) (Fig. 1). In 2015, species richness, also decreased significantly compared to 2013: $t = 3.25$, $P = 0.006$ and 2014: $t = 5.73$, $P < 0.001$ (Fig. 1).

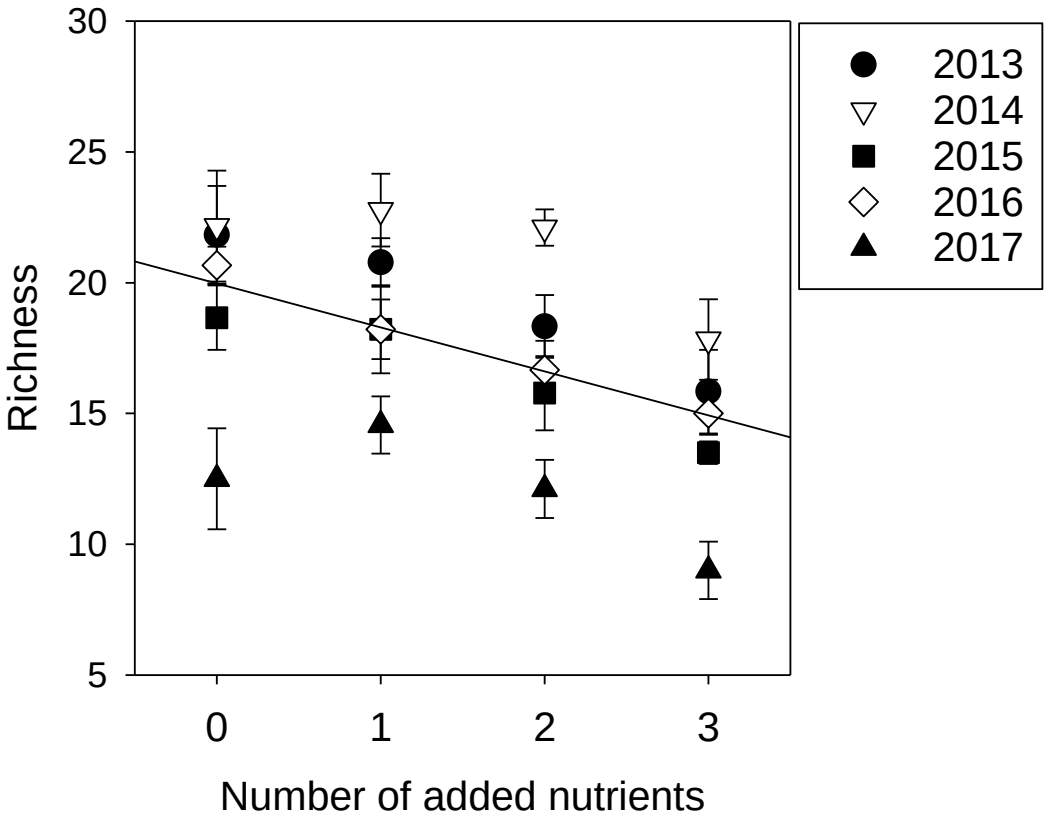

Fig. 1 Species richness along an increasing number of added nutrients, for 2013 - 2017. Symbols represent mean ± SEM, for each year. For controls (0) and three added nutrients $n = 6$; for one and two added nutrients $n = 9$. Line shows linear regression for all data between richness and number of added nutrients.

Total productivity

Adding nutrients increased significantly grassland productivity ($F_{3,149} = 18.68$, $P < 0.001$; linear regression: $F_{1,149} = 74.98$, $P < 0.001$), which also varied significantly across years ($F_{4,149} = 38.06$, $P < 0.001$). However, there was a significant interaction between nutrient treatment and year ($F_{12,149} = 4.20$, $P < 0.001$). Indeed, in 2015 (disturbance year) there were no significant differences in productivity between nutrient treatments (Fig. 2). Only addition of three nutrients had consistently (except in 2015) a significant positive effect on productivity compared to one added nutrients (2013: $t = 5.76$, $P < 0.001$; 2014: $t = 5.56$, $P < 0.001$; 2016: $t = 6.05$, $P < 0.001$; 2017: $t = 5.96$, $P < 0.001$) and controls (2013: $t = 4.93$, $P < 0.001$; 2014: $t = 5.54$, $P < 0.001$; 2016: $t = 5.20$, $P < 0.001$; 2017: $t = 5.43$, $P < 0.001$). Productivity was significantly lower in 2015 compared to the other years, for two added nutrients (2013: $t = 6.34$, $P < 0.001$; 2014: $t = 6.36$, $P < 0.001$; 2016: $t = 6.11$; $P < 0.001$; 2017: $t = 3.51$, $P = 0.003$) and three added nutrients (2013: $t = 8.92$, $P < 0.001$; 2014: $t = 7.76$, $P < 0.001$; 2016: $t = 7.24$; $P < 0.001$; 2017: $t = 6.46$, $P < 0.001$) (Fig. 2). However, for controls and one added nutrient treatments, productivity in 2015 was only significantly lower than in 2013 (high productivity year) (controls: $t = 3.73$, $P = 0.003$; one added nutrients: $t = 3.42$, $P = 0.009$) (Fig. 2).

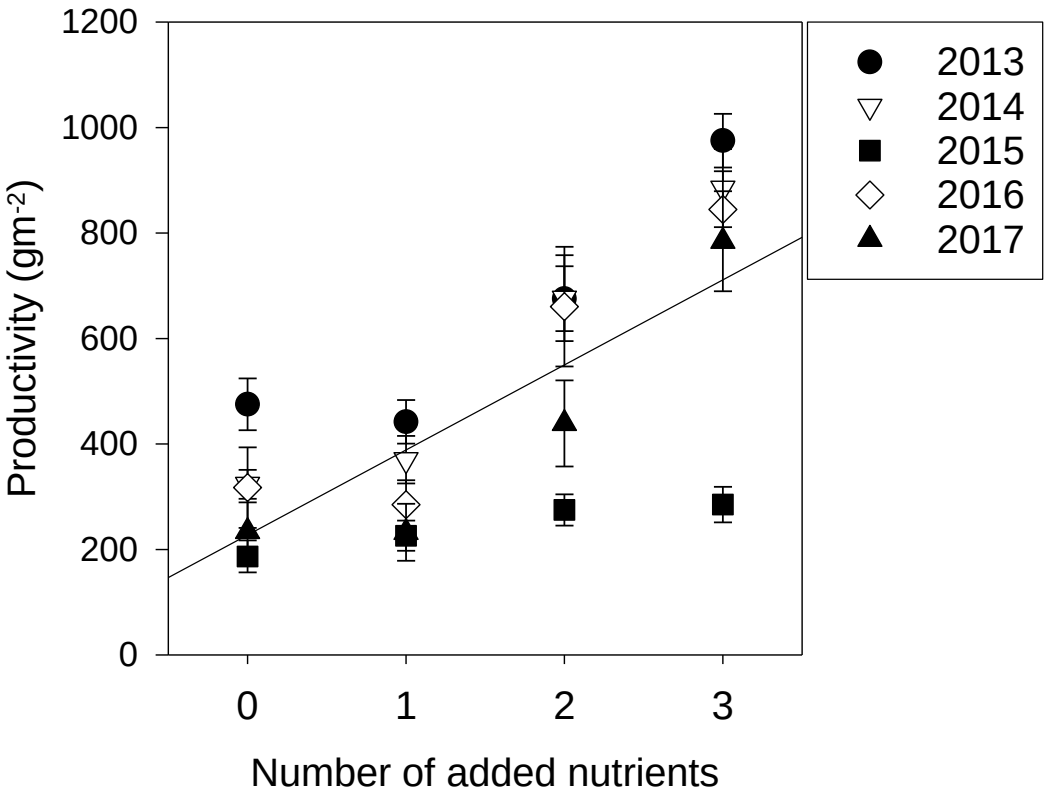

Fig. 2 Productivity along an increasing number of added nutrients, for 2013 - 2017. Symbols represent mean ± SEM of productivity for each year. For controls (0) and three added nutrients *n* = 6; for one and two added nutrients *n* = 9. Line shows linear relation between number of added nutrients and productivity.

Drivers of productivity

Best fit model for total productivity explained 54% of its variation ($F_{4,199}$ = 35.80, P < 0.001) and included number of added nutrients (β = 0.401, P < 0.001), spring SPEI (β = 0.525, P < 0.001), previous year productivity (β = 0.292, P < 0.00) and number of years with nutrient addition (β = 0.205, P = 0.006) (Table S1; Supporting information).

Functional group composition

Proportion of forbs and graminoids was significantly affected by the number of added nutrients (forbs: $F_{3,149}$ = 6.73, P = 0.002; graminoids: $F_{3,149}$ = 25.39, P < 0.001). Overall, forbs were negatively and graminoids positively correlated to increasing number of added

nutrients (linear regression: $F_{1,149} = 24.11$, $P < 0.001$; $F_{1,149} = 37.64$, $P < 0.001$, for forbs and graminoids, respectively) (Fig. 3a, b). Both functional groups were affected by year (forbs: $F_{4,149} = 24.33$, $P < 0.001$; graminoids: $F_{4,149} = 25.72$, $P < 0.001$) and by a significant interaction between number of added nutrients and year (forbs: $F_{12,149} = 3.74$, $P < 0.00$; graminoids: $F_{12,149} = 2.09$, $P = 0.024$).

In 2013 and 2017, controls presented the highest forb proportion, especially compared to three nutrients treatment (2013: $t = 5.13$, $P < 0.001$; 2017: $t = 4.76$, $P < 0.001$) (Fig. 3a). Interestingly, in 2015, forb proportion registered a high increase in three nutrients treatment to the detriment of graminoids compared to 2013 ($t = 4.76$, $P < 0.001$), 2016 ($t = 2.70$, $P = 0.032$) and 2017 ($t = 6.23$, $P < 0.001$).

In three out of five years, graminoid proportion was consistently higher in three nutrients treatment compared to controls (2013: $t = 5.72$, $P < 0.001$; 2016: $t = 3.54$, $P = 0.003$ and 2017: $t = 4.60$, $P < 0.001$) (Fig. 3b).

Legume proportion was not significantly affected by number of added nutrients ($F_{3,149} = 0.36$, $P = 0.784$) (Fig. 3c), but varied significantly across years ($F_{4,149} = 27.12$, $P < 0.001$).

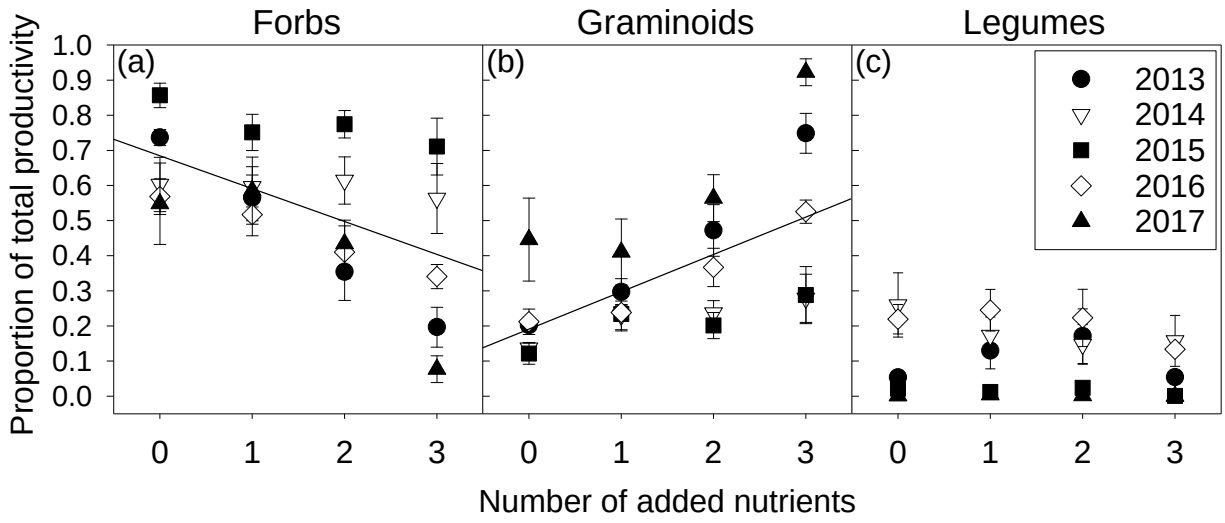

Fig. 3 Proportion of forbs (a), graminoids (b) and legumes (c) along an increasing number of added nutrients for 2013 - 2017. Symbols represent mean ± SEM of proportion of productivity for each year. For controls (0) and three added nutrients $n = 6$; for one and two added nutrients $n = 9$. Lines show linear relation, for all data, between number of added nutrients and functional group proportion in the five years.

Drivers of functional group composition

Forbs responded negatively (β = -0.232, *P* = 0.003) and graminoids positively (β = 0.381, *P* < 0.001) to added nutrients, but for legumes, nutrient addition was not included in the best fit model (Table 2). Grazing affected negatively graminoids (β = -0.341, *P* < 0.001), but positively forbs (β = 0.783, *P* < 0.001) (Table 2). While graminoids responded negatively (β = -0.529, *P* < 0.001) and legumes positively (β = 1.294, *P* < 0.001) to full growing season SPEI, forbs were positively affected by winter SPEI (β = 0.424, *P* < 0.001) (Table 2). Forbs and legumes responded positively to previous year abundances (forb: β = 0.184, *P* = 0.020, and legumes: β = 0.259, *P* < 0.001), also number of years with nutrient addition affected positively legumes (β = 0.835, *P* < 0.001) (Table 2).

Table 2 Models explaining the inter-annual variation in current-year functional group proportion. Each row corresponds to the best stepwise regression model for each functional group based on AIC (Akaike information criterion – lowest AIC) (Table S1; Supporting information). Nutrients refers to number of added nutrients, fall SPEI (September, October and November), spring SPEI (February, March and April) and winter SPEI (December and January). Growing season SPEI integrates the whole of the growing season, from September to April.

Best model	***F*-value $_{(DF)}$**	***P* - value**	**R^2 adj.**	**AIC**
Forbs = grazing + nutrients + winter SPEI +previous forbs	$20.28_{(4.119)}$	<0.001	0.39	-389.99
Graminoids = nutrients + grazing + growing season SPEI	$40.32_{(3.119)}$	<0.001	0.49	-424.94
Legumes = growing season SPEI + nutrient addition years + previous legumes	$28.58_{(3.119)}$	<0.001	0.41	-507.81

Effects of N, P and K on functional group proportion

Forbs were negatively and significantly related to the presence of P in treatments, but not to N and K (Table 3). Legumes were negatively and significantly related to N and positively and significantly affected by P (Table 3). Graminoids were positively and significantly related to the presence of N and P (Table 3).

Table 3 Multi-linear regression coefficients using N, P, and K as binary independent variables and proportion of functional groups (forbs, graminoids and legumes) as dependent variables. (DF) refers to degrees of freedom. *P*-value: *<0.05, **<0.001.

	N	P	K	*F*-value $_{(DF)}$	*P* - value	R^2 adj.
Forbs	-0.021	-0.253***	-0.01	$16.50_{(3,149)}$	< 0.001	0.24
Graminoids	0.110***	0.151***	-0.01	$13.44_{(3,149)}$	< 0.001	0.20
Legumes	-0.165***	0.089**	0.013	$10.81_{(3,149)}$	< 0.001	0.17

Community stability

In 2015, a very dry winter and spring combined with heavy defoliation by cattle in February critically affected grassland productivity and functional group composition (Figs. 2 and 3). Pre-grazing LAI (January 27, 2015) showed significant differences among nutrient treatments ($F_{3,29} = 27.60$, $P < 0.001$). Three added nutrients had a significantly higher LAI (1.57 ± 0.26), compared to two nutrients (0.53 ± 0.17; $t = 4.95$, $P < 0.001$); one nutrient (0.10 ± 0.02; $t = 8.05$, $P < 0.001$) and controls (0.07± 0.01; $t = 7.80$, $P < 0.001$). LAI of two nutrients was also significantly different from one nutrient ($t = 3.47$, $P = 0.004$) and controls ($t = 3.59$, $P = 0.004$) with no significant difference between these last two treatments ($t = 0.49$, $P = 0.63$). Differences in grassland LAI after grazing (March 6, 2015) were similar to those observed before grazing: three added nutrients still had a significant higher LAI (0.08 ± 0.02) compared to two nutrients (0.03 ± 0.01; $t = 3.27$, $P = 0.012$), one nutrient (0.02 ± 0.003; $t = 4.82$, $P < 0.001$) and controls (0.02 ± 0.004; $t = 3.86$, $P = 0.003$), with no differences between controls and one nutrient ($t = 0.59$, $P = 0.561$); however, two nutrients treatment was not significantly different from one ($t = 1.74$ $P = 0.257$) and controls ($t = 0.97$ $P = 0.57$).

In 2015, productivity decreased, as compared to 2014, an average of 41% for controls and one added nutrient treatments, and 59% and 68% for two and three added nutrient treatments, respectively. Grassland productivity resistance to the 2015 disturbance was significantly and negatively affected by the number of added nutrients ($F_{3,29} = 5.71$, $P = 0.004$) (Fig.4a). Addition of three nutrients reduced resistance compared to controls ($t = 2.94$, $P = 0.034$) and one added nutrient treatments ($t = 3.79$, $P = 0.005$) (Fig. 4a). Resistance of three nutrients was on average 27% lower than controls and one nutrient treatment. Interestingly, resistance to the disturbance events was positively and

significantly related to average species richness of the previous years (2013 and 2014) ($F_{3,29} = 5.65$, $P < 0.001$). Recovery was positively and significantly affected by the number of added nutrients ($F_{3,29} = 5.41$, $P = 0.005$; Fig.4b). Differences were only significant between one and three added nutrients, with the former having the highest recovery 48.07% ± 6.45 while one added nutrient had only 8.38% ± 6.04 ($t = 3.74$, $P = 0.006$) (Fig.4b). Resilience was not affected by the number of added nutrients ($F_{3,29} = 0.07$, $P = 0.976$; Fig. 4c). One year after the 2015 disturbance event, i.e. in 2016, productivity was 77.49 % ± 6.84 of pre-disturbance productivity, in 2014. Grassland stability, calculated as the CV for the whole of the study period (5 years) was also not affected by added nutrients ($F_{3,29} = 0.23$, $P = 0.875$).

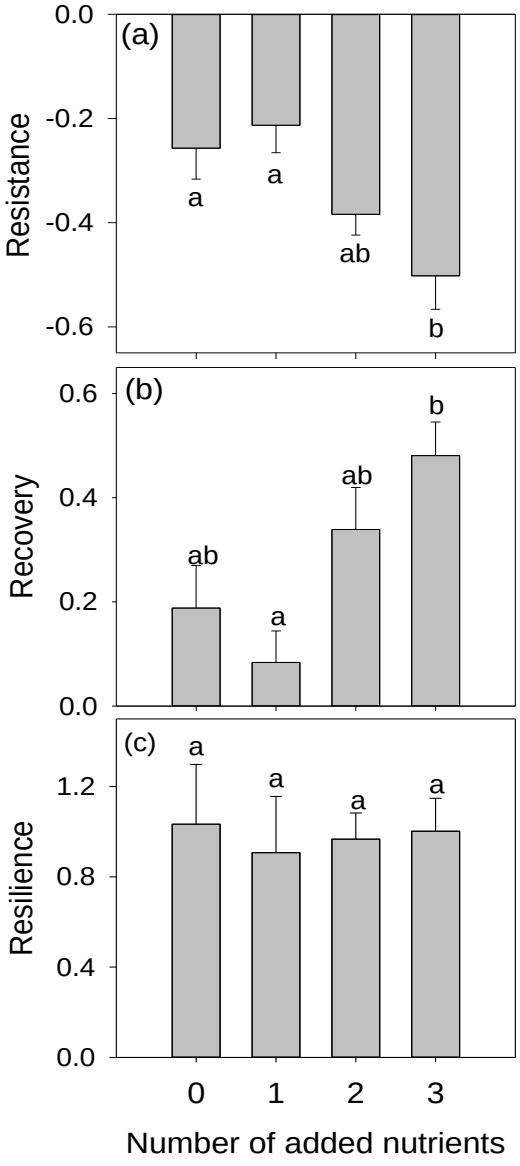

Fig. 4 Resistance (a), recovery (b) and resilience (c) of productivity along an increasing number of added nutrients. Bars represent mean ± SEM. For controls (0) and three added nutrients $n = 6$; for one and two added nutrients $n = 9$. Different letters indicate significant differences between number of added nutrients.

4.5 Discussion

Our study identified major effects of increased number of added nutrients on Mediterranean grassland diversity and productivity. Decreasing nutrient limitation increased productivity while consistently decreased species richness across different climatological years. Functional group composition, however, responded interactively to nutrient addition and prevailing climatic conditions. The addition of nutrients did not affect resilience (or stability) of the grassland productivity even when subjected to a disturbance event, a combination of grazing and drought. Resilience was offset by decreased resistance and increased recovery after the disturbance event along increased number of nutrients.

Grassland productivity

Our results demonstrated that besides precipitation, grassland productivity was co-limited by multiple nutrients, as shown for other grasslands around the world (Fay et al., 2015; Harpole et al., 2016). Grassland productivity was dependent on the number of added nutrients, spring SPEI, previous year productivity and number of years with nutrient addition. In Mediterranean grasslands, maximum growth occurs in spring, when longer days, mild temperatures and precipitation accelerate vegetation growth (Xu and Baldocchi, 2004). Spring precipitation is also critical for extending the growing season (La Pierre et al., 2016), which explains the relevance of spring SPEI for grassland productivity. Although aboveground biomass was removed each year in summer, at the end of the growing season, the low but significant contribution of previous year productivity may have resulted from increased soil organic matter content, improved water holding capacity or nutrient input (e.g. Oesterheld et al., 2001; Dudney et al., 2017). After five years of nutrient addition, productivity of our grassland still responded to nutrient addition, except in 2015, the year of the disturbance event.

Precipitation modulated the response of productivity to increasing number of added nutrients (Knapp and Smith 2001; Huxman et al., 2004), which can be expected in Mediterranean regions (Churkina and Running, 1998). Productivity, overall, increased from drier to wetter years. However, even in the 2017 dry year, productivity increased in the

three added nutrients treatment. In contrast, in a N limited Mediterranean grassland in Israel, productivity response to added N was negligible under low precipitation (Seligman and Van Keulen, 1989). We found that water and nutrients co-limited productivity in our grassland, which has commonly been observed in Mediterranean ecosystems (Hooper and Jonhson, 1999; Zavaleta et al., 2003; Dukes et al., 2005; Harpole et al., 2007).

Functional group proportion

In our study, reducing nutrient limitation led to a significant increase in graminoids and a decrease in forbs, as observed in other studies (e.g. DiTommaso and Aarssen, 1989; Zavaleta et al., 2003; Harpole et al., 2007; DeMalach et al., 2017). Positive graminoid response to nutrient addition was however, more consistent throughout the five years than the negative response of forbs. A negative response of forbs to nutrient addition or inconsistent in certain years, such as we and others have observed, (e.g. Zavaleta et al., 2003; Harpole et al., 2007; Buis et al., 2009) may be due to the high number of species and trait diversity that forbs encompass determining different responses to nutrients (Craine et al., 2001; Zavaleta et al., 2003). We also found that functional groups responded differently to timing, distribution, and amount of precipitation, as seasonal SPEI explained partially functional group variability.

Graminoids were more responsive to increased nutrient addition and outcompeted forbs, possibly because of their greater increase in root cation exchange capacity (Woodward et al., 1984; DiTommaso and Aarssen, 1989) and higher root density (Craine et al., 2001). Due to these traits and others such as high stature or specific leaf area (Ansquer et al., 2009; Craine and Dybzinski, 2013), graminoids can easily outcompete shorter species, or species with a more conservative nutrient and water use such as forbs (DiTommaso and Aarssen, 1989; Zavaleta et al., 2003; Niu et al., 2014).

A clear negative effect of nutrients on forbs, observed in the wetter 2013 and the drier 2017 years, suggests that forb productivity is driven by complex interacting effects of climate, nutrient availability, as well as their competitive ability. In 2014 and 2015, forbs were the dominant functional group in all nutrient treatments, representing on average 59% and 77% of productivity in all treatments, respectively, outcompeting graminoids. Forbs were possibly favored by the high fall and winter precipitation as reflected by the

integration of winter SPEI in the best fit model (Espigares and Peco, 1993; Peco and Espigares, 1994; Nogueira et al., 2017). Additionally, heavy grazing in February 2015, must have contributed to relieve forbs from graminoid competition, as herbivores remove more easily taller species such as graminoids than some forbs (Knapp et al., 2012; Peco et al., 2012).

Although the number of nutrients did not affect legume abundance, legumes were favored by P and PK, but not by N, as observed in other studies (e.g. DiTommaso and Aarssen, 1989; Suding et al., 2005; van den Berge et al., 2010). Legume proportion was highest in average precipitation years (2014 and 2016) but lowest in wet (2013) and dry (2017) years as found by others (Caldeira et al., 2001; Grant et al., 2014). High precipitation in fall and winter, combined with average lower temperatures, could have explained higher legumes proportion in 2014, as legume species avoid germinating under high temperatures even when water is available, as a protection against a false break (Del Pozo and Aronson, 1999; Nogueira et al., 2017). Interestingly, legumes responded positively to the number of years with nutrient addition, however this also coincides with years of grazing exclusion. Legumes have a high nutritional value and are preferentially selected by cattle (e.g. Rochon et al., 2003), which can explain their higher abundances with increasing number of years of grazing exclusion. In the same way, in 2015, heavy grazing, in combination with drought, affected legumes negatively.

Species richness and stability

Grassland richness decreased with nutrient addition (Gough et al., 2000; Harpole et al., 2007; Isbell et al., 2013; Harpole et al., 2016). Contrary to other studies, however, environmental conditions did not interact with nutrient availability to affect species richness (Flores-Moreno et al., 2016; Smith et al., 2016). During the 2017 dry year, species richness decreased significantly, as reported by others (e.g. Tilman and El Haddi, 1992; Miranda et al., 2009), but in a similar way in all nutrient treatments (average 36% decrease in species richness per treatment). Drought can limit germination of many species (Tilman and El Haddi, 1992) or have a differential effect on seedling mortality (Espigares and Peco, 1995).

Observed decreased species richness with nutrient addition is in line with the prediction that the supply of co-limiting resources can reduce niche dimensionality and result in community species loss (Harpole and Tilman, 2007; Harpole et al., 2016). For example, increased nutrient availability can favor fast-growing species that competitively exclude slow-growing species with more conservative nutrient use strategies (Chapin, 1980; Harpole and Tilman, 2006). Increased productivity with increased nutrient addition may lead to light limitation in the understory, a mechanism that contributes to explain diversity loss in response to nutrient addition (Hautier et al., 2009).

In our study, grassland stability, as estimated by CV, was not affected by increased nutrients, although communities with added nutrients were also less species rich (22% less species on average). Our results therefore suggest a null relationship between stability and number of species. Positive diversity-stability relationships have been shown in communities with similar nutrient availability across a gradient of species richness (e.g. Tilman et al., 1997; Caldeira et al., 2005). However, when resources including nutrient availability differ between communities this relationship may not hold (Hautier et al., 2014). When analyzing the diversity–stability relationships from 41 natural grasslands in fertilized and unfertilized plots, Hautier et al. (2014) concluded that the reduction in diversity due to fertilization was not the main driver of stability effects. This was also observed in other studies (e.g. Grman et al., 2010; Xu et al., 2015) where nutrient addition affected stability through a combination of effects on seasonal production variability and species asynchrony (Hautier et al., 2014). Also, Grman et al. (2010) found that greater dominance of some species in fertilized plots can contribute to maintain stability in spite of decreased species richness. Nonetheless, we found that communities with higher species richness (and low added nutrients) were more resistant to combined grazing and drought, in accordance to the diversity-stability hypothesis that states that more diverse communities are more likely to contain species that can overcome disturbances (Yachi and Loreau, 1999; Caldeira et al., 2005; Tilman et al., 2014). Species-rich grasslands may also display a high number of species performing similar functions (i.e., functional redundancy), thus the loss of a redundant species may still ensure a certain level of functional diversity and ecosystem functioning (Petchey and Gaston, 2006; Cadotte et al., 2011; Rota et al., 2017).

Community responses to drought and grazing disturbance

In 2015, the disturbance year, productivity in the three added nutrient treatment decreased by 67% as compared to the average productivity of the other four years. As for controls, productivity in 2015 was only significantly lower than the wet year productivity in 2013. These results show that grasslands with higher nutrient availability were less resistant to the disturbance events.

The higher LAI in three added nutrient treatments, early in the growing season (January 2015), as compared to controls, probably made those communities more susceptible to defoliation. Indeed, although all treatments were defoliated, the relative decrease in LAI in three added nutrients was 30% higher than controls, as a denser and taller canopy can be preferred by cattle (Knapp et al., 2012; Peco et al., 2012). Also, nutrient addition may have increased species palatability (e.g. Mattson, 1980). Low species richness in communities with more nutrients may also have contributed to their low resistance to herbivory and drought as discussed earlier.

Despite being less resistant, communities with more nutrients and lower species richness were better able to recover from the disturbance event. Although, several studies have reported a positive effect of species richness on recovery (e.g. van Ruijven and Berendse 2010; Vogel et al. 2012), our results are consistent with those of Kreyling et al. (2017), that observed that recovery was independent of species richness in high-productive communities. In our nutrient-limited grassland, greater nutrient availability may have favored more competitive species such as graminoids that were able to take advantage of increased resource availability, after the stressful events and thus outcompete species with low resource-retaining strategies. Resistance and recovery responses to the disturbance events cancelled out each other determining a null effect on community resilience in all nutrient treatments in accordance with the stability (CV) results.

It is important to understand how interacting global changes affect ecosystem functioning and resilience to disturbances. Many studies on grassland productivity and diversity addressed climate variability and single nutrient additions (N), but very few have investigated how multiple nutrient additions interact with climatic variability. Our Mediterranean grassland, located in a hotspot region for climate changes, was co-limited

by multiple nutrients and water, and responded to added nutrients through increased productivity and loss of species richness. Although decreased species richness may imply lower resistance, it may also stimulate higher recovery after major disturbances in nutrient-enriched communities. A combined grazing and drought disturbance negatively affected productivity of mainly nutrient enriched grasslands. Changes in land management and higher frequency of severe droughts, will negatively affect Mediterranean grassland functioning and resilience under nutrient enriched scenarios. Lower species richness and added nutrients favoring graminoid dominance may in the long-term decrease seed bank diversity and ultimately Mediterranean grassland resilience to extreme droughts and combined disturbances.

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CHAPTER 5

Nutrient addition and drought interact to change the structure and decrease the functional diversity of a Mediterranean grassland

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5 Nutrient addition and drought interact to change the structure and decrease the functional diversity of a Mediterranean grassland

5.1 Abstract

Anthropogenic activities are increasing nutrient availability and altering precipitation regimes. This may lead to critical changes in grasslands functioning and resilience. This is particularly important for grasslands in the Mediterranean Basin that have evolved in nutrient poor soils, and where more frequent and prolonged droughts are projected to occur. However, there is limited knowledge regarding the interacting effects of multiple nutrient inputs and rainfall variability on Mediterranean grassland functional structure and diversity.

We conducted a nutrient addition experiment during four contrasting precipitation years in a Mediterranean grassland. We established four treatments that varied in the number of added nutrients from no added nutrients (control), to one added nutrient (Nitrogen-N, Phosphorus-P or Potassium-K), two added nutrients, (NP, NK or PK) and three added nutrients (NPK). We assessed the effect of increasing nutrient addition in wet, normal, dry and very dry years on species functional traits at the community level. We determined the community functional structure (e.g. Community Weighted Mean, CWM) and functional diversity (e.g. Functional Dispersion, FDis) for eight key functional traits indicators of nutrient and water use strategies. We also assessed if CWM, FDis and species richness were related to the aggregate grassland functioning property, productivity.

We found that CWM was affected by nutrient addition and precipitation and, for some traits, by their interaction. However, FDis of most traits was affected by precipitation. The very dry year had a negative effect on FDis of most traits (e.g. dispersal modes, nutrient uptake strategies) and interacted with three added nutrients to decrease FDis of growth-forms. Conversely, FDis of reproductive traits decreased during the wet year. Species richness and FDis were not related to grassland productivity, whereas CWM was the main determinant of grassland productivity supporting the importance of species functional traits in determining ecosystem functioning.

Our results highlight drought as a critical factor determining a decrease in the functional diversity of Mediterranean grasslands. Moreover, drought can also interact with nutrient addition changing the dominance of many traits and further decreasing functional diversity. This will have important implications for ecosystem functioning and resilience in the context of global changes.

Keywords: community weighted mean; drought; functional dispersion, functional diversity; inter-annual climatic variability; Mediterranean grassland; nutrient addition; plant functional traits.

5.2 Introduction

Anthropogenic activities, such as fossil fuel combustion and fertilizer applications are increasing nutrient inputs, such as of nitrogen (N) and phosphorus (P), into the biosphere (Peñuelas et al., 2013). Mediterranean grasslands are species-diverse ecosystems of high economic and ecological value (Hector and Bagchi, 2007; Bugalho and Abreu, 2008) likely to experience biodiversity changes due to interacting global change drivers (Sala et al., 2000).

Nutrient addition often leads to decreases in grassland plant species diversity and compositional shifts, which may potentially affect ecosystem functioning and resilience (Bobbink et al., 2010; Stevens et al., 2010; Harpole et al., 2016; Flores-Moreno et al., 2016). Climate change may also lead to reduction in species richness (e.g. Thuiller et al., 2015) and induce changes in grassland community composition (e.g. Dornelas et al., 2014). Moreover, these two drivers of change, may interact, further affecting grassland diversity and functioning (e.g. Zavaleta et al., 2003; Harpole et al., 2007). This is particularly important in the Mediterranean region, a hot-spot for climate changes (Giorgi, 2006), where increased precipitation variability and frequency of droughts is forecasted to occur (Costa et al., 2012; Kovats et al., 2014).

Many studies have evaluated the responses of grassland plant species composition and diversity to alterations in environmental conditions, such as fertilization or drought (e.g., Tilman and El Haddi,1992; Gough et al., 2000; Zavaleta et al., 2003; Harpole et al., 2007;

2016; Bobbink et al., 2010; Stevens et al., 2010; DeMalach et al., 2017). Species richness and diversity, per se, however do not reflect the species role in ecosystem functioning (e.g. Tilman et al., 1997; Díaz and Cabido, 2001; Mason et al., 2005; McGill et al., 2006; Violle et al., 2007; Cadotte et al., 2011; Mouillot et al., 2011), while species functional traits, can provide a more mechanistic and predictive understanding of ecosystem responses to environmental changes (e.g. Díaz et al., 2007; Mouillot et al., 2011). Functional traits are morphological, physiological or phenological features of species that can be measured at the individual level, which determine the species functional role in ecosystems and/or their response to changing environmental conditions or disturbances (Díaz and Cabido, 2001). Ecosystem functioning and resilience depend on the functional traits of the dominant species in a community (i.e. community functional structure), in accordance with the mass-ratio hypothesis (Grime, 1998), and on the dissimilarity of functional trait values within that community (here defined as functional diversity *sensu* Laliberté and Legendre, 2010). Communities with a high functional diversity are expected to improve ecosystem functioning by increased complementarity in resource use among species (Tilman et al., 1997) and to provide high resilience to disturbances (Díaz and Cabido, 2001; Mason et al., 2005; Díaz et al., 2007; Cadotte et al., 2011; Mouillot et al., 2011; Volaire et al., 2014; Valencia et al., 2015).

Although the response of communities' functional structure and diversity to decreased nutrient limitation (e.g. Suding et al., 2005; Janeček et al., 2013, Helsen et al., 2014) or to drought (e.g. Polley et al., 2013; Carmona et al., 2015; Nunes et al., 2017) have been previously assessed, the concurrent effects of both remain unclear. Nutrient addition was shown to favor the abundance of species with high specific leaf area (SLA) and height, probably because these traits correlate better with competition for nutrients and light (Westoby et al., 2002; Suding et al., 2005; Ordoñez et al., 2009; Janeček et al., 2013; Eskelinen and Harrison, 2015). This may explain a positive response in the abundance of grasses (Zavaleta et al., 2003) to the detriment of the less competitive, rosette growth-forms (Pfestorf et al., 2013) when nutrients were added. The increased frequency of droughts predicted for the Mediterranean Basin, coupled with its high inter-annual precipitation variability, may also modify the functional structure and diversity of grassland communities, and affect ecosystem functioning (Pérez-Camacho et al., 2012; Carmona et al., 2015; Nunes et al., 2017). For example, drought generally favors small stress-tolerant and slow-growing species with low SLA, high leaf dry matter content (LDMC) (e.g. Westoby et al., 2002; Carmona et al., 2015), delayed flowering onset and shorter flowering

duration (Crimmins et al., 2013), although an earlier flowering onset has also been observed with increasing drought (Pérez-Camacho et al., 2012; Shavrukov et al., 2017). In addition, Rota et al. (2017) observed a decrease in seed mass functional diversity due to drought, whereas functional diversity of SLA was unresponsive.

These findings indicate that nutrient addition and drought can select for different traits underlying opposite strategies, such as high and low SLA, respectively. This draws attention to the importance of understanding the response of grassland communities to interacting global change drivers such as increased nutrient availability and drought (Bernard-Verdier et al., 2012; Pérez-Ramos et al., 2013; Eskelinen and Harrison, 2015; Rota et al., 2017). However, few studies have assessed how nutrient addition and water limitation interact in Mediterranean grasslands to affect community functional structure and trait diversity and thus, ecosystem functioning and resilience.

In this four-year study, we assessed how increasing nutrient availability and different precipitation years, ranging from wet to very dry years, interacted to affect community functional structure and functional trait diversity of a Mediterranean grassland. To achieve this, we focused on eight key plant functional traits known to be responsive to nutrient and water use strategies (Cornelissen et al., 2003, Garnier et al., 2007) and used the community weighted mean (CWM) and functional dispersion (FDis) as metrics of community functional structure and functional diversity, respectively. We hypothesized that (1) nutrient addition and precipitation are strong interacting filters of plant community functional structure; and (2) as precipitation plays a fundamental role in determining richness and community composition in Mediterranean ecosystems, functional diversity will be primarily influenced by precipitation.

5.3 Materials and Methods

Site Description

We conducted our study in a semi-natural Mediterranean grassland, located north-east of Lisbon, Portugal (38^{o}49´45.13´´N, 8^{o}47´28.61´´W). The grassland community, occasionally grazed by cattle until 2012, was dominantly composed by annual C3 species that grow in winter and early spring, and start senescing in late spring. Overall, dominant species are the forbs *Tolpis barbata* L., *Plantago bellardii* All., the graminoids *Agrostis pourretii* Willd.,

Avena barbata Link, and the nitrogen-fixing legumes *Ornithopus compressus* L. and *Trifolium arvense* L.

The climate is Mediterranean-type with long- term (1961-1990) mean annual rainfall of 709 mm, with considerable inter-annual variations. Mean annual temperature is 15.9 °C, ranging from 10 ºC in January to 22.5 ºC in August (INMG, 1991). Site topography is flat. The soil is a well-drained deep Haplic Arenosol (IUSS, 2006) with a low soil water retention capacity and N content (0.12 %).

Experimental Design

We added N, P and potassium (K) combined with micronutrients to 5 x 5 m plots, following a full factorial combination in a complete randomized three block design. We considered four treatments that varied in the number of added macronutrients (0, 1, 2 and 3) following Harpole et al. (2016). Hereafter, we use the term 'number of added nutrients' for simplicity. Specifically, the treatments were: Controls, i.e. no added nutrients, termed as 0 (6 replicates); additions of one single nutrient N, P or K (9 replicates); addition of two join nutrients (NP, NK or PK), named as 2 added nutrients (9 replicates); and plots fertilized with a combination of 3 nutrients, NPK that is 3 added nutrients treatment (6 replicates). The experiment is part of the global Nutrient Network experiment (www.nutnet.org; Borer et al., 2017)

The experiment started in 2012 and lasted until 2017. However, we excluded the year of 2015 as the cattle broke through the fence during February and heavily defoliated and trampled the plots. We added nutrients every fall, at the beginning of the growing season. We applied N, P, and K at a rate of 10 g N m^{-2} yr^{-1}. N was added from 2012 to 2016 as slow-release urea (60-90 days), switching in 2017 to two separate additions of urea (3 months apart to mimic slow release) due to restricted availability of timed-release urea. P was added as triple-super phosphate and K as potassium sulfate. Micronutrients (6% Ca, 3% Mg, 12% S, 0.1% B, 1% Cu, 17% Fe, 2.5% Mn, 0.05% Mo, and 1% Zn) were added at a rate of 100 g m^{-2} with K and were only applied in the 2012, the first year of the study, to avoid micronutrient toxicity.

The site was fenced to avoid intense cattle grazing and trampling. We used a brush cutter and removed all standing vegetation in the fenced experimental area at the end of each

growing season after seed production and dispersal to eliminate potential effects of carried-over biomass (Dudney et al., 2017).

In *situ* precipitation was recorded using a tipping bucket rain gauge (RG2, Delta-T Devices, Cambridge, UK), connected to a CR1000 and AM16/32B multiplexer data logger (Campbell Scientific, Logan, USA).

Precipitation Years

During our study, the hydrological precipitation of the four years was 483 mm (very dry year, 2017), 598 mm (dry year, 2016), 708 mm (normal year, 2013) and 814 mm (wet year, 2014) (Figure 1). The year of 2017 was the driest hydrological year, with precipitation 32% below the long-term average (709 mm), 2016 was 16% below the long-term average while 2013 was similar to the long-term average and 2014 the wettest with the precipitation 15% above the long-term average (Fig. 1). The year of 2015, which was not included in our analysis, was also a very dry year, especially during winter and spring, with total precipitation 465 mm, 34% below the long-term average.

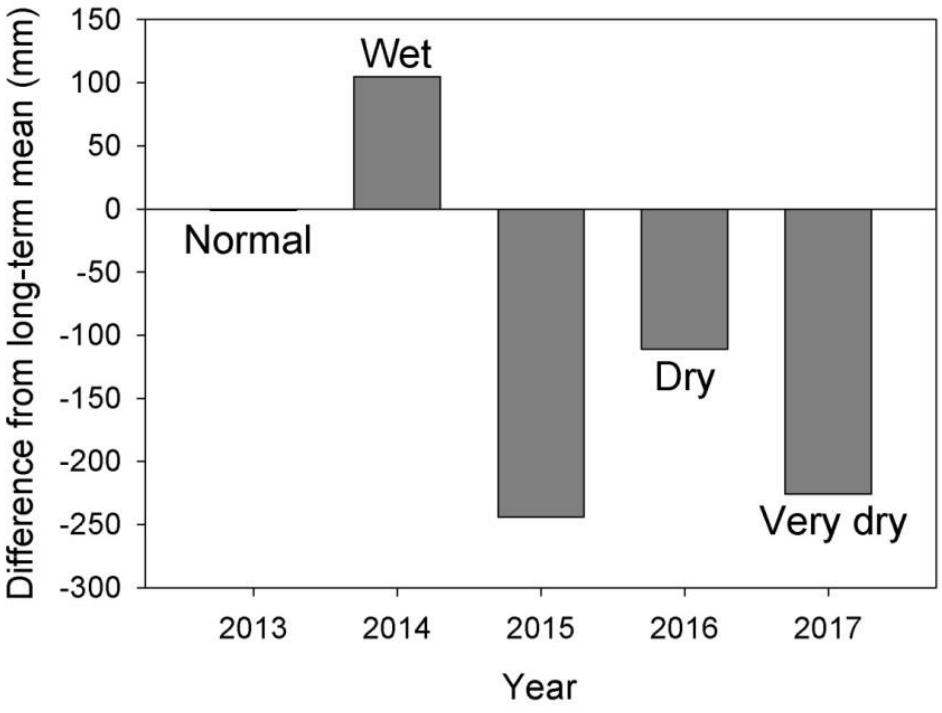

Fig. 1 Precipitation difference from the long-term hydrological year average (709 mm, 1961-1990), for 2013 to 2017. 2013 (normal precipitation year – total precipitation, 708 mm), 2014 (wet year –

814 mm), 2015 (very dry year – 465 mm) not included in our study; 2016 (dry year –598 mm) and 2017 (very dry year – 483 mm).

Vegetation Sampling

Species cover (%), to the nearest 1%, of each plant species was estimated annually by observation, using a modified Daubenmire method (Daubenmire, 1959), at peak vegetation growth, in a permanently marked $1m^2$ quadrat in each plot.

Species Richness and Aboveground biomass

We have also examined the pattern of plant species richness and grassland productivity to increasing number of added nutrients (Nogueira et al., submitted 2018). Species richness was determined as the number of plant species per plot each year. Aboveground plant biomass was estimated by collecting, every year, all individual plants rooted within two 10 cm x 100 cm strips per plot, at the time of peak biomass accumulation. We considered aboveground biomass as a proxy for productivity. The locations of the clipping strips were moved within each plot every year to prevent clipping effects. Samples were dried to constant mass at 60 °C prior to weighing.

Trait Selection

Selection of plant functional traits is a critical step to understand and predict community functioning and responses to environmental changes and disturbance (Petchey and Gaston, 2006). Therefore, we selected eight plant traits including continuous, ordinal and categorical traits, that reflect plant functional responses to water and soil nutrient availability (e.g. Cornelissen et al., 2003) (Table 1).Trait data was obtained for 61 species identified in our grassland communities throughout the 4-year study. Trait information was obtained through direct observation or measurements in the field, following standard protocols (Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2013), and/or derived from the literature (Franco, 1971, 1984; Valdés et al., 1987; Garnier et al., 1997; Castroviejo, 2012; Paula and Pausas, 2013) (Table 1;). We used a single mean trait value per species

to compute functional trait metrics. If different trait values for the same species could be found in the literature, we opted for plant trait values obtained in Mediterranean regions. Although intraspecific trait variability plays an important role in many ecosystem processes and contributes to the understanding of community response to environmental changes (Lepš et al., 2011), in this study we only consider differences in functional structure and functional diversity caused by changes in species composition (Bernard-Verdier et al., 2012; Carmona et al., 2015; Rota et al., 2017).

Table 1 Functional traits studied, their typology / units and their main role in plant functioning. Superscript number next to each trait indicates sources used to obtain trait information: [1]observed or measured in the field following standard protocols (Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2013) and derived from bibliographic sources; [2] only derived from bibliographic sources.

Trait	Type	Typology/units	Functional role
Vegetative			
Growth-form[1]	Categorical	Ascending Climber Erect Graminoid Prostrate Rosette	Light capture, above-ground competition, resistance to disturbance
Maximum height[1]	Continuous	cm	Light capture, above-ground competition
Leaf			
SLA[1]	Continuous	$mm^2.mg^{-1}$	Light capture, growth rate
LDMC[1]	Continuous	$mg.g^{-1}$	Physical resistance, stress tolerance
Reproductive			
Onset flowering[2]	Semi-quantative	Initial month number	Phenological, reproductive strategy, drought-tolerance
Duration flowering[2]	Semi-quantative	Number of months	
Regenerative			
Dispersal strategy[2]	Categorical	Anemochory Barochory Ectozoochory Endozoochory	Dispersal ability, covered distances
Below-ground			
Nutrient uptake strategy[2]	Categorical	N-fixer Orchid root Root hemiparasite None specified	Uptake of essential nutrients

Functional Trait Metrics

To assess community functional structure, we determined the Community Weighted Mean (CWM) for each trait, which corresponds to the average trait value in a community, weighted by the relative abundance of the species carrying each trait value (Garnier et al., 2007). CWM trait values are a quantitative translation of the biomass ratio hypothesis (Grime, 1998) by reflecting the dominant traits values in a community. For continuous traits CWM values represent the mean value of that trait in the community, for categorical and binary traits CWM values correspond to the proportion of each category in the community.

Functional trait diversity within the plant community was determined through Functional Dispersion (FDis), which is closely related to Rao's quadratic entropy (Laliberté and Legendre, 2010). It estimates the dispersion of species in functional trait space, weighted by their relative abundances. It is calculated as the weighted mean distance, in multidimensional trait space, of individual species from the weighted centroid of all species, where weights correspond to species relative abundances (Laliberté and Legendre, 2010). The minimum value FDis can take is 0 (e.g. communities composed by only one species) and it has no upper limit. FDis has several advantages over other functional diversity indices as it takes into account species relative abundances, it is unaffected by species richness, handles any number and type of traits (including more traits than species) and is not strongly influenced by outliers (Laliberté and Legendre, 2010). We calculated FDis for each functional trait individually and for the six traits combined, that responded significantly to nutrient addition and precipitation. The calculation of trait metrics was done using the FD package (Laliberté and Shipley, 2011), in software R (R Core Team, 2015).

Statistical Analyses

To examine the effect of number of added nutrients and precipitation (hydrological year, October to September) on community plant species composition, we performed a non-metric multidimensional scaling ordination (NMDS) based on species cover of each plot

sampled in each nutrient addition treatment throughout our four-year study. We used *meta MDS* function from vegan package (Oksanen et al., 2013), with Bray-Curtis distance measure. Ordination *stress* statistic was used as a measure of goodness of fit. A permutational multivariate analysis of variance (PERMANOVA) was performed with 9999 permutations on Bray-Curtis communities' dissimilarities, to test for the effect of number of added nutrients and precipitation year (Oksanen, 2011) on species composition.

The influence of number of added nutrients, precipitation year, and their interaction on single-trait CWM and FDis values and on FDis computed for traits that were significantly affected by nutrient addition and precipitation years was examined by means of general linear mixed models. Number of added nutrients and precipitation year were used as fixed effects explanatory variables and plots were used as a random factor, to account for non-independent observations made along the four years of the study in each plot. Tuckey pairwise comparisons with Bonferroni corrections were performed to check for differences between number of added nutrients and between different precipitation years.

We used Spearman correlation to test for the significance of correlations between species richness, CWM, FDis and productivity. We selected growth-form, maximum height, SLA and LDMC for Spearman correlation as these traits may reflect grassland productivity (Table 1). Spearman correlation was used to account for possible nonlinear relationships.

All the analyses were performed under R statistical environment (R Core Team, 2015). Graphs, except Figure1, were done with Sigmaplot 13.0 (Systac Software, Inc., San Jose, USA).

5.4 Results

The Effect on Community Species Composition

Main gradients in community species composition are described by a 2-dimensional non-metric multidimensional scaling (NMDS) ordination with a final stress value of 0.22 (Figure 2a, b). The first axis (NMDS1) explained 35.5% and the second axis (NMDS2) explained 18.6% of the variation in species composition (Figure 2a, b). Plant community composition changed significantly with the increase in number of added nutrients (PERMANOVA test: *F*

= 9.98, $P < 0.001$), and with the total hydrological precipitation, mainly in the very dry year (PERMANOVA test: $F = 14.01$, $P < 0.001$).

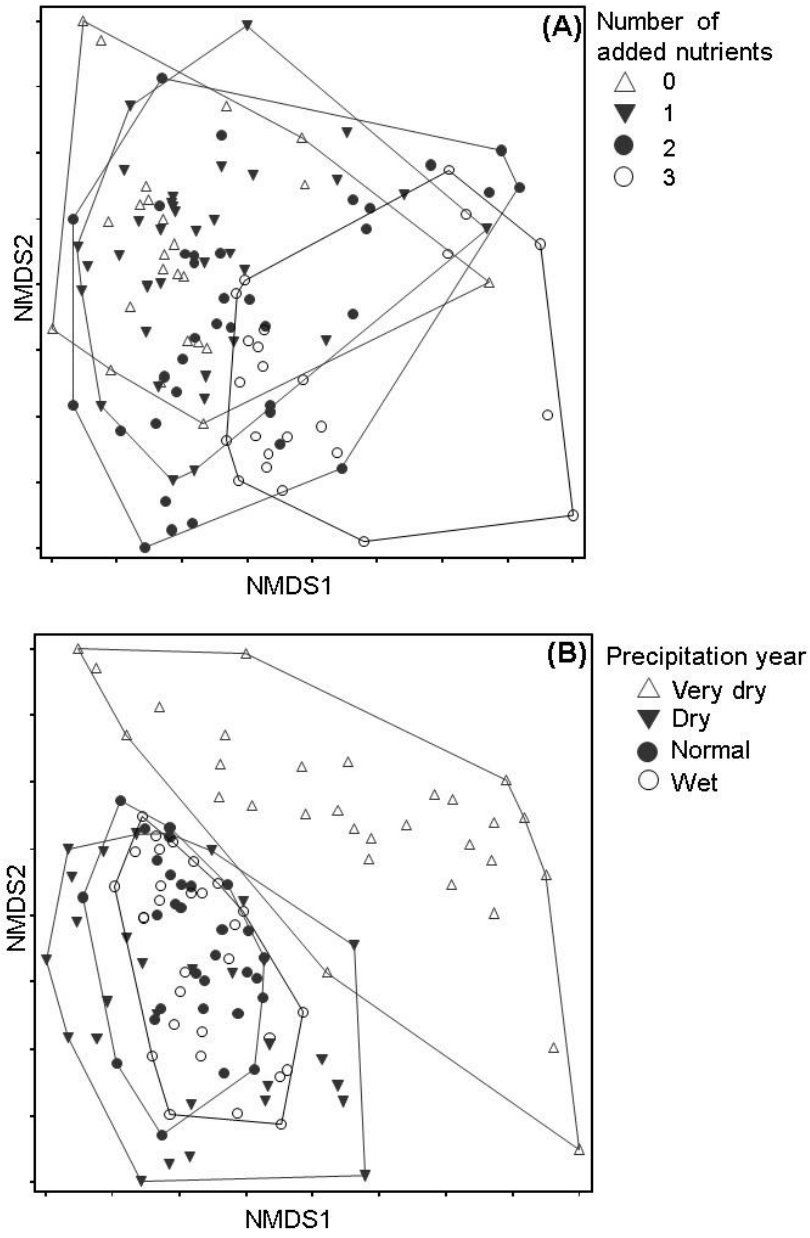

Fig. 2 Non-metric multidimensional scaling (NMDS) based on species cover, with points representing plots sampled in each nutrient addition treatment in different hydrological precipitation years: normal year (2013 – 708 mm), wet year (2014 – 814 mm), dry year (2016 – 598 mm) and very dry year (2017 – 483 mm). Points representing plots are united according to (**A**) the number of added nutrients; (**B**) hydrological year. Final stress for the 2-dimensional configuration was 0.22.

Functional Structure of Plant Communities

Ten out of sixteen CWM trait values were significantly related to number of added nutrients (Table 2), four of which had a significant interaction with precipitation year (graminoid and erect growth-forms, SLA and LDMC) (Table 2). Differences between nutrient addition treatments were, in most cases, only significant between three added nutrients and controls and one added nutrient treatment. In general, CWM values were significantly affected by the very dry year compared to other type of years (Table 2).

Table 2 Statistical results of the linear mixed models for CWM for each functional trait. Number of added nutrients and precipitation year, as well as their interactions, were used as fixed effects explanatory variables. Plots were used as a random factor. Significant results ($P < 0.05$) are in bold. Superscript signs (-) or (+) indicate a negative or positive effect of explanatory variables.

		Number added nutrients			**Precipitation**			**Number added nutrients x Precipitation**		
Trait	**Category**	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Vegetative										
Growth-form	Ascending	1	5.86	**0.022**$^{(-)}$	1	14.74	**<0.001**$^{(+)}$	1	0.65	0.421
	Graminoid	1	54.91	**<0.001**$^{(+)}$	1	55.55	**<0.001**$^{(-)}$	1	8.51	**0.045**$^{(-)}$
	Rosette	1	12.2	**0.002**$^{(-)}$	1	15.43	**<0.001**$^{(+)}$	1	1.56	0.215
	Erect	1	15.11	**<0.001**$^{(-)}$	1	15.4	**<0.001**$^{(-)}$	1	14.99	**<0.001**$^{(+)}$
	Prostrate	1	0.45	0.507	1	49.13	**<0.001**$^{(+)}$	1	0.06	0.804
Maximum height		1	21.53	**<0.001**$^{(+)}$	1	9.55	**0.003**$^{(+)}$	1	2.17	0.144
Leaf										
SLA		1	9.56	**0.005**$^{(+)}$	1	1.07	0.3035	1	6.22	**0.015**$^{(-)}$
LDMC		1	14.93	**0.001**$^{(+)}$	1	51.96	**<0.001**$^{(-)}$	1	5.9	**0.017**$^{(-)}$
Reproductive										
Onset flowering		1	10.63	**0.003**$^{(-)}$	1	54.94	**<0.001**$^{(-)}$	1	3	0.087
Duration flowering		1	0.02	0.88	1	85.42	**<0.001**$^{(+)}$	1	8.52	**0.005**$^{(+)}$
Regenerative										
Dispersal mode	Anemochory	1	0.04	0.86	1	36.15	**<0.001**$^{(-)}$	1	0.95	0.332
	Barochory	1	0.01	0.907	1	44.08	**<0.001**$^{(+)}$	1	1.72	0.193
	Ectozoochory	1	10.31	**0.003**$^{(-)}$	1	13.75	**0.000**$^{(+)}$	1	0.04	0.834
	Endozoochory	1	3.03	0.093	1	9.49	**0.003**$^{(+)}$	1	2	0.16
Nutrient uptake	N-fixing	1	0.13	0.716	1	53.84	**<0.001**$^{(+)}$	1	0.38	0.538
	Root-hemiparasite	1	40.72	**<0.001**$^{(-)}$	1	14.28	**<0.001**$^{(+)}$	1	0.05	0.832

Species Growth-forms and Height

Overall, dominant species in our grassland belonged to the growth-form of graminoids (proportional cover, average ± s.e.m.: 0.34 ± 0.02), erect (0.32 ± 0.02) and rosette (0.21 ± 0.02), while ascending (0.03 ± 0.003) and prostrate species (0.10 ± 0.01) were less abundant.

Increasing number of added nutrients had a significant positive effect on the cover of graminoids to the detriment of ascending, erect and rosette growth-forms (Table 2). Abundance of prostrate species was only significantly and positively affected by precipitation and unaffected by nutrient addition (Table 2). In the very dry year, prostrate species were residual in the community (0.01 ± 0.003) as compared to other years (0.13 ± 0.01, average across treatments).

Graminoids were affected by a significant interaction between the number of added nutrients and precipitation (Table 2). During the wet year, abundance of graminoids was lowest (0.17 ± 0.02) compared to other years (0.40 ± 0.03); with no significant differences between nutrient addition treatments (Figure 3a). However, for other precipitation years, graminoids were significantly more abundant in the three added nutrients treatment as compared to one added nutrient (normal: t = -3.89, P = 0.004; dry: t = -3.48, P = 0.011; very dry: t = -5.73, P < 0.001) and controls (normal: t = -4.26, P = 0.001; dry: t = -4.09, P = 0.002; very dry: t = -4.89, P < 0.001) (Table 2; Figure 3a). Abundance of species with a rosette or ascending growth-form were significantly and negatively affected by number of added nutrients and positively affected by precipitation (Table 2). Contrary to graminoids, rosette species were less abundant in three nutrients treatment (0.10 ± 0.021) compared to one nutrient (0.26 ± 0.03; z = -4.64, P < 0.001), two nutrients (0.19 ± 0.03; z = -2.76, P = 0.035), and marginally to controls (0.25 ± 0.02; z = -2.50, P = 0.076). Rosette species were also, marginally significantly more abundant during the wet year (0.30 ± 0.03) compared to the very dry year (0.19 ± 0.04) (z = 2.50, P = 0.074). Abundance of erect species was only significantly lower in three nutrients treatment (0.05 ± 0.03) compared to one nutrient (0.29 ± 0.09; t = 3.73, P = 0.006) and controls (0.43 ± 0.16; t = 4.30, P = 0.001) during the very dry year (Table 2).

We found significantly higher maximum height in communities where three nutrients were added (average species height: 86.93 cm ± 3.19) compared to one nutrient treatment (64.32 cm ± 2.28; z = 3.27, *P* = 0.007) and controls (61.13 cm ± 3.73; z = 4.33, *P* < 0.001), with a significant positive precipitation effect (Table 2; Figure 3b).

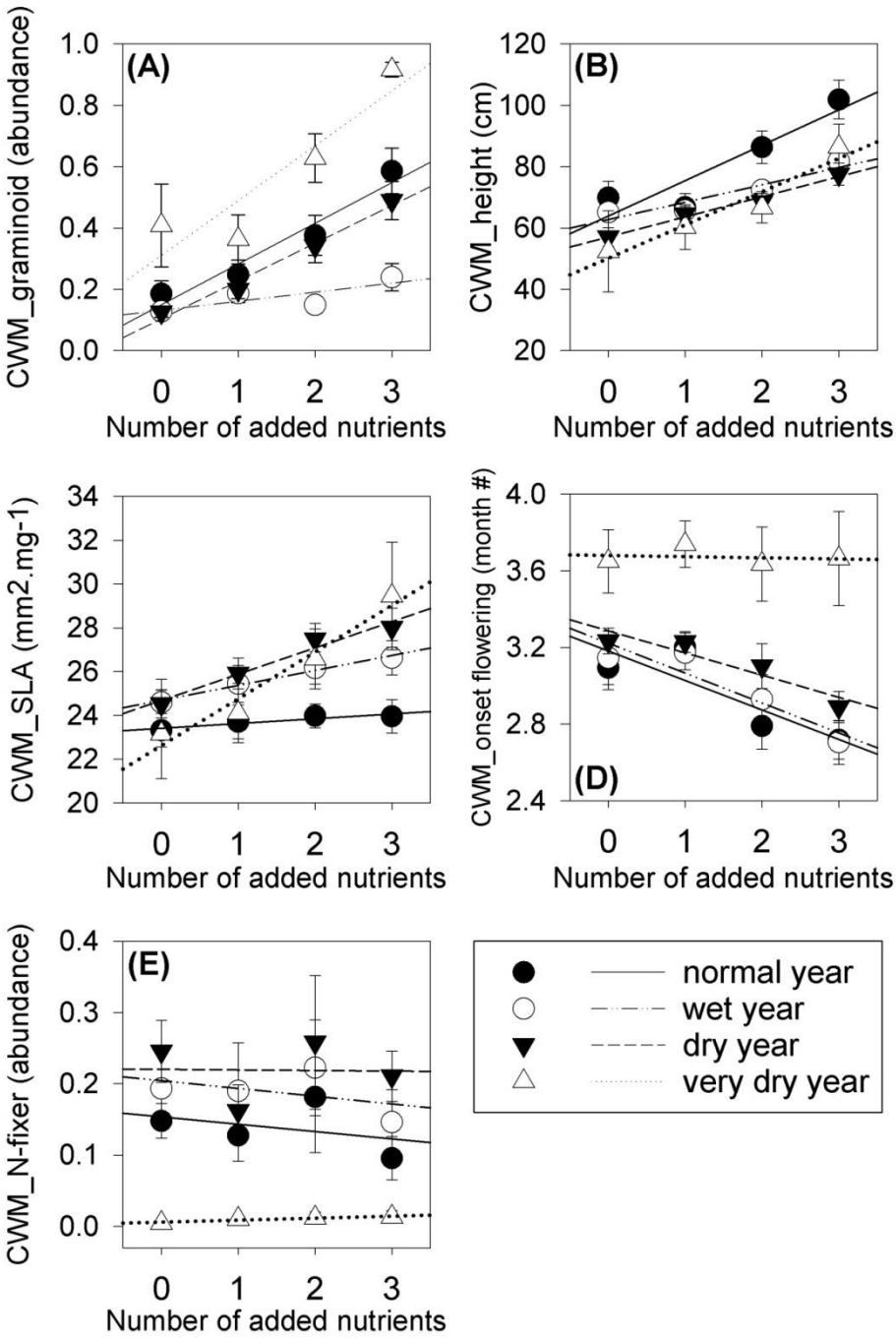

Fig. 3 CWM values for (A) graminoid growth-form; (B) height; (C) SLA; (D) onset of flowering and (E) N-fixing ability, along a gradient of number of added nutrients, for a normal precipitation year (2013 – 708 mm), wet year (2014 – 814 mm), dry year (2016 – 598 mm) and very dry year (2017 –

483 mm). Symbols represent mean ± s.e.m, for number of added nutrients for each precipitation year. For one and two added nutrients n = 9; for three added nutrients n = 6 for each precipitation year. Lines show mean response between CWM trait values and number of added nutrients for each precipitation year.

Species Leaf Traits

SLA was the only trait with no significant association with precipitation (Table 2; Figure 3c). However, the nutrient addition treatments only significantly affected CWM of SLA during the very dry year, with the three added nutrients exhibiting higher SLA (29.45 mm^2 mg^{-1} ± 2.46) compared to one nutrient treatment (24.11 mm^2 mg^{-1} ± 1.35) (z = 3.30, P = 0.002) and controls (23.15 mm^2 mg^{-1} ± 2.03) (z = 3.63, P = 0.006) (Table 2; Figure 3c). LDMC was also higher in three nutrients (301.83 mg g^{-1} ± 15.31) compared to one nutrient treatment (227.32 mg g^{-1} ± 20.86) (t = -4.13, P = 0.002) and to controls (236.62 mg g^{-1} ± 16.57) (t = -3.16, P = 0.024) during the very dry year. There were no differences in LDMC between treatments for other precipitation years, although precipitation had a significant negative effect on LDMC (Table 2).

Species Reproductive Strategies

Across all treatments and precipitation years, onset of flowering in our grassland occurred between February and April, and was significantly affected by the number of added nutrients and precipitation (Table 2; Figure 3d). Overall our grassland community responded with an earlier onset of flowering with increasing number of added nutrients. Communities had later flowering during the very dry year compared to normal (t = -2.84, P = 0.027) and wet years (t = -2.84, P = 0.061). Flowering duration, which varied overall between 3 and 6 months, was shorter during the very dry year compared to the wet year (z = 4.01, P <0.001), normal year (z = 4.83, P <0.001) and dry year (z = 3.72, P = 0.001). Also, only in the very dry year communities with three added nutrients had shorter flowering duration than one (t = 3.15, P = 0.025) and two nutrients treatments (t = 3.14, P = 0.025).

Species Dispersal Strategies

Across all nutrient treatments and precipitation years, our grassland community was dominated by species with wind dispersal (anemochorous species) (0.71 ± 0.02), followed by species with dispersal by gravity (barochorous) (0.26 ± 0.01); and rarely by internal animal transport (endozoochorous) (0.02 ± 0.003) and external animal transport (ectozoochorous species) (0.01 ± 0.002). Dispersal modes were unaffected by number of added nutrients (Table 2). Abundance of species with wind dispersal was highest during the very dry year (0.89 ± 0.02) compared to the, wet (0.63 ± 0.03; z = -3.92, P <0.001), normal (0.71 ± 0.03; z = - 4.16, P <0.001) and the dry year (0.62 ± 0.03; z = -5.33, P < 0.001), while species with dispersal by gravity or internal animal transport were significantly less abundant (Table 2).

Species Nutrient Uptake Strategies

Most species in our grassland were non N-fixers (0.83 ± 0.015, average across treatments and years). Abundance of N-fixing species was not significantly affected by number of added nutrients (Table 2), and represented 0.14 ± 0.015 (average across treatments and years) of our grassland community. N-fixing species were, however significantly negatively affected by very low precipitation, compared to the wet year (z = 6.60; P <0.001), normal year (z = 5.82, P <0.001) and dry year (z = -7.64, P < 0.001) (Figure 3e). During the very dry year, N-fixing species abundance decreased by 94%; from 0.18 ± 0.017 (three-year average) to 0.01 ± 0.003 (very dry year). Although, abundance of hemi-parasites species was low in our grassland, it was further decreased in the three nutrients treatment (0.01 ± 0.005) than in controls (0.09 ± 0.012), during the normal (t = 4.92, P <0.001) and the dry years (t = 5.92, P <0.001).

Functional Dispersion of Traits (FDis)

The functional dispersion of growth-form was significantly negatively affected by nutrient addition (Table 3), but only in the very dry year, with three nutrients having the lowest diversity in growth-form compared to the other treatments, two added nutrients (t = 5.91, P

< 0.001); one added nutrients (t = 7.11, P < 0.001) and controls (t = 4.12, P = 0.002) (Figure 4a). FDis of growth-form was significantly lower during the very dry year compared to other years (wet: z = 4.27; P <0.001, normal: z = 5.13, P <0.001 and dry year: z = 4.21, P < 0.001). FDis of most traits were not affected by increasing number of added nutrients but they were significantly affected by precipitation year (Table 3). FDis of height trait, increased overall with precipitation, whereas, FDis of the onset of flowering and flowering duration decreased with precipitation (Table 3, Figure 4b and c). FDis of dispersal mode was significantly lower during the very dry year (0.15 ± 0.02) compared to the wet (0.30 ±0.01; z = 4.41, P < 0.001), normal (0.27±0.01; z = 4.48, P < 0.001) and the dry year (0.31± 0.01; z = 5.04, P < 0.001). FDis of nutrient uptake strategy followed the same trend and was significantly lower during the very dry year compared to the wet (z = 8.15, P < 0.001), normal (z = 9.29, P < 0.001) and the dry year (z = 10.07, P < 0.001) (Table 3; Figure 4d). FDis of SLA and LDMC were unaffected by nutrient addition and precipitation (Table 3). The FDis of the combined six traits (multi-trait FDis) decreased significantly during the very dry year in all treatments compared to the remaining years (wet: z = 5.99, P < 0.001; normal: z = 6.75, P < 0.001 and dry year: z = 7.05, P < 0.001) but it was not affected significantly by the number of added nutrients (Table 3; Figure 4e).

Table 3 Statistical results of the linear mixed models analyzing the effects of nutrient addition and precipitation on functional dispersion (FDis) for each functional trait and for a multi-trait combination of six relevant traits. Number of added nutrients and precipitation years, as well as their interactions, were used as fixed effects explanatory variables. Plots were used as a random factor. Significant results (P < 0.05) are in bold. Superscript signs (-) or (+) indicate a negative or positive effect of explanatory variables.

	Number added nutrients			**Precipitation**			**Number added nutrients x Precipitation**		
Trait	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Growth-form	1	6.42	**0.017**$^{(-)}$	1	44.72	**<0.001**$^{(+)}$	1	5.09	**0.027**$^{(+)}$
Height	1	0.6	0.444	1	7.04	**0.009**$^{(+)}$	1	0.01	0.911
SLA	1	1.63	0.213	1	0.01	0.926	1	0.02	0.886
LDMC	1	0	0.984	1	3.1	0.082	1	0.43	0.514
Onset flowering	1	1.42	0.243	1	19.56	**<0.001**$^{(-)}$	1	0.02	0.881
Duration flowering	1	0.09	0.772	1	9.4	**0.003**$^{(-)}$	1	0.55	0.461
Dispersal mode	1	1.88	0.182	1	34.47	**<0.001**$^{(+)}$	1	1.09	0.299
Nutrient uptake	1	3.01	0.094	1	67.31	**<0.001**$^{(+)}$	1	0.05	0.832

Multi-trait	1	3.39	0.076	1	53.93	**<0.001**$^{(+)}$	1	0.642	0.425

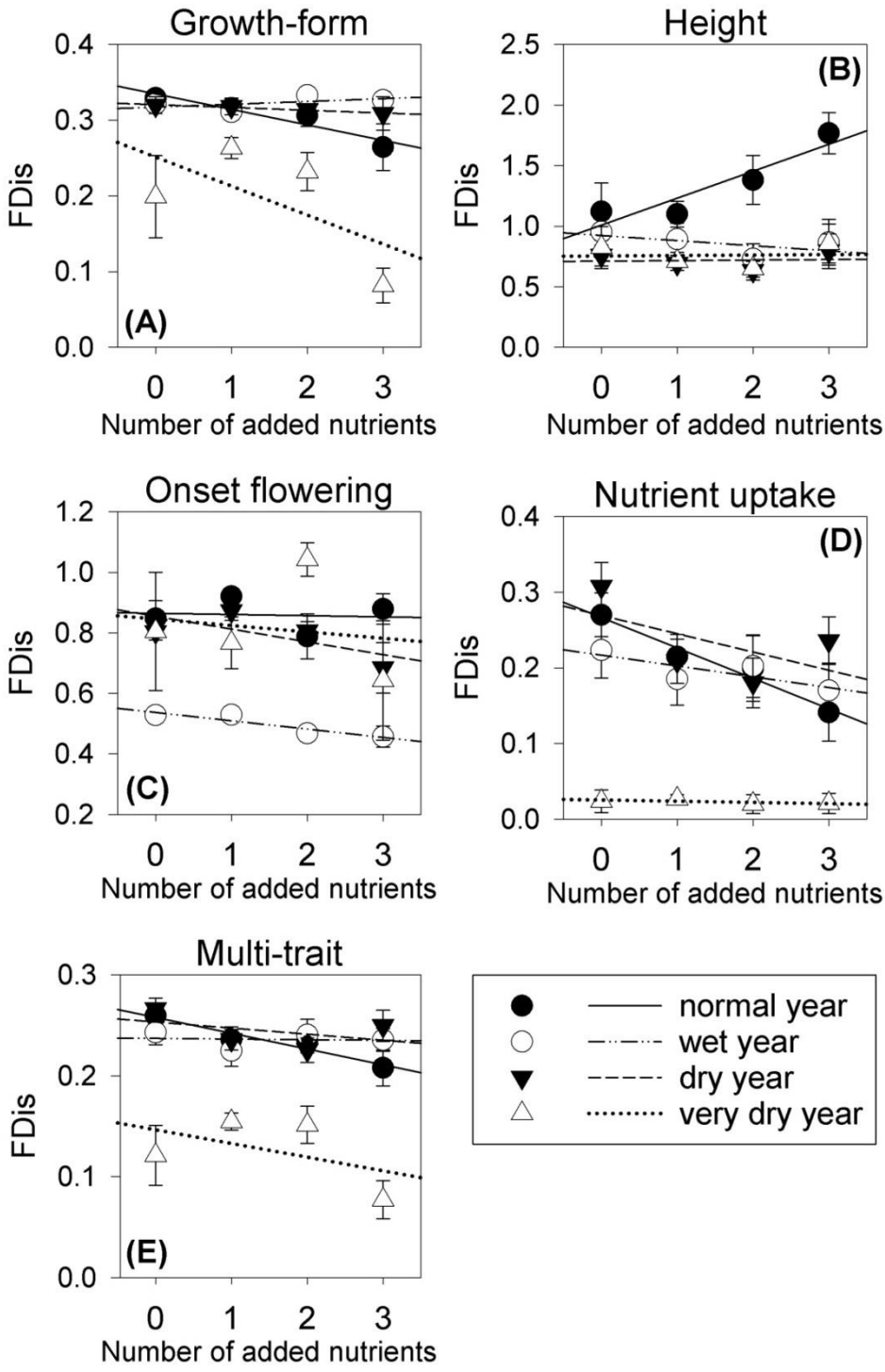

Fig. 4 FDis values for (**A**) growth-form; (**B**) height; (**C**) onset of flowering; (**D**) nutrient uptake strategy and (**E**) multi-traits, the combined six traits affected by nutrient addition and precipitation, along a gradient of number of added nutrients, for a normal precipitation year (2013 – 708 mm),

wet year (2014 – 814 mm), dry year (2016 – 598 mm) and very dry year (2017 – 483 mm). Symbols represent mean ± s.e.m, for number of added nutrients for each precipitation year. For one and two added nutrients *n* = 9; for three added nutrients *n* = 6 for each precipitation year. Line shows mean response between FDis values for each trait/ multi-trait and number of added nutrients, for each precipitation year.

Functional Traits and Productivity

Increasing number of added nutrients significantly decreased species richness and significantly increased productivity as reported in Nogueira et al. 2018 (submitted). However, species richness had no significant relationship with productivity (ρ = -0.046, *P* = 0.621; *n* = 120), whereas most of the CWM trait values were significantly related to productivity (Table 4). Higher abundance of graminoid and prostrate species were significantly positively related to productivity, as were species with a higher maximum height, or/and higher LDMC. Contrarily, high abundance of species with an erect or rosette growth form was significantly negatively related to productivity (Table 4). FDis of the analyzed traits had no relation with productivity (Table 4).

Table 4 Spearman correlations (ρ) between CWM (community weighted mean) and functional dispersion (FDis) of each trait and productivity (*n* = 120; for all treatments and for the four years). Significant results (*P* < 0.05) are in bold.

		CWM		**FDis**	
Trait	**Category**	**Spearman ρ**	***P***	**Spearman ρ**	***P***
Vegetative					
Growth-form	Ascending	0.001	0.995	0.075	0.412
	Graminoid	0.33	**<0.001**		
	Rosette	-0.256	**0.005**		
	Erect	-0.317	**<0.001**		
	Prostrate	0.242	**0.008**		
Maximum height		0.643	**<0.001**	0.119	0.194
Leaf					
SLA		0.157	0.086	0.095	0.300
LDMC		0.194	**0.034**	-0.074	0.420

5.5 Discussion

Overall, our results showed a combined effect of nutrients and precipitation on the community functional trait structure (CWM), while functional diversity (FDis) was mainly affected by precipitation. Nutrient addition only decreased functional diversity of growth-forms in the very dry year. A clear change in species composition was observed during the very dry year and in three nutrients addition treatment, which was reflected by marked changes in community functional structure and trait diversity.

Precipitation was the major environmental filter determining CWM and FDis. This is in agreement with the high inter-annual precipitation variability and low soil fertility context in which Mediterranean annual grasslands have coevolved (Noy-Meir, 1973; Peco et al., 1998; Pérez-Camacho et al., 2012; Carmona et al., 2015; Rota et al., 2017). Functioning and resilience of the Mediterranean grasslands is probably mostly based on a high functional diversity of traits responsive to different levels of soil water availability combined with resource conservative functional traits (Pérez-Camacho et al., 2012; Carmona et al., 2015). The decrease in co-limitation by water and nutrients increased the dominance of traits associated with resource-acquisition such as maximum height (Westoby et al., 2002, Eskelinen and Harrison, 2015) but not SLA. However, graminoids responded positively to nutrient addition as found in other studies (Hautier et al., 2009; Spasojevic and Suding, 2012; Harpole et al., 2016; Helsen et al., 2014; Niu et al., 2017) but negatively to precipitation (but see Harpole et al., 2007). Graminoids have higher root cation exchange capacity compared to most dicots (Woodward et al., 1984; DiTommaso and Aarssen, 1989) and higher root density (Craine et al., 2001) which makes them highly competitive for available nutrients. Interestingly, in the wet year, a low abundance year for graminoids, the functional group did not respond to nutrient availability increase. On the contrary, during the very dry year, graminoids were very abundant; increasing overall two fold comparing to other years, and were better competitors for nutrients. This was also supported by the increase in SLA and LDMC in the three added nutrient communities in the very dry year. In accordance, Wellstein et al. (2017), in a Mediterranean grassland, found that graminoids had a higher SLA under experimental drought and demonstrated better growth performance, which was most likely related to their strategy to allocate resources to roots. Additionally, in our study, the FDis of growth-forms decreased with decreasing precipitation, being lowest in the very dry year, particularly in communities with lower co-limitation by nutrients. The niche dimension hypothesis (Harpole and Tilman,

2007) states that the decrease in the number of limiting resources should lead to loss of diversity. However, in our study it was during the very dry year, with a high limitation by soil water, that a decrease in FDis of growth-forms, dispersal modes, nutrient uptake strategies, as well as FDis of the main six functional traits combined, was observed. Contrastingly, during the wet year, with the highest soil water availability, only the FDis of onset and duration of flowering decreased. Our results indicate that the mechanisms underlying functional diversity loss can be dependent on the environmental and evolutionary context.

In our study, the increase in species height and SLA in three added nutrients communities probably resulted from the increase of graminoids which were taller and had higher SLA than rosette and erect growth-forms. This pattern was also observed by Ansquer et al. (2009) in grassland communities in the Pyrenees. Dominance of graminoids with increased nutrients may also help to explain the earlier flowering in nutrient rich communities, except in the very dry year. Later flowering during the very dry year is somewhat counterintuitive. If abundance of graminoids increased during the very dry year, onset of flowering should have been earlier. Also, early flowering is one of the many plant strategies to cope with increased water limitation (e.g. Shavrukov et al., 2017). However, in our study, *Vulpia bromoide*s was the dominant graminoid species during the very dry year in all treatments. This species has a later onset of flowering and a shorter flowering duration which may explain main reproductive strategy observed in the communities during that year. During a wet year, we found a reduction in FDis of reproductive traits, which suggests that trait filtering, may also occur under more favorable conditions (Bernar-Verdier et al., 2012).

Species in our grassland were mostly dispersed by wind and gravity. Although nutrient addition had no effect on observed plant dispersal modes, drought affected wind dispersal positively and gravity dispersal negatively. The higher abundance of graminoids, all wind-dispersal species and the significant reduction of N-fixing species, most of them gravity-dispersal species, during the very dry year explains this result. In Mediterranean environments with low and highly fluctuating precipitation, small or large seeds can be both optimal (Volis and Boher, 2013). However, the larger seed production and greater longevity in the seed bank associated with small seeded species can be advantageous with increased aridity (Volis and Boher, 2013).

Nutrient uptake strategies mainly separated N-fixing species from other species. The very dry year had a negative effect on the abundance of N-fixing species as observed in other studies (e.g. Espigares and Peco, 1993, 1995; Del Pozo and Aronson, 1999). Also, N-fixing species were found to be highly vulnerable to drought during their reproductive phase, which may shorten the duration of reproductive development, reducing seed number and weight (Daryanto et al., 2015). As N-fixing species have, generally, a positive effect on the nitrogen budget of grasslands (Spehn et al., 2002), the projected increase in the frequency and intensity of droughts in the Mediterranean region, can negatively affect the nitrogen budget. Although in our study there was no association between added nutrients and abundance of N-fixing species, we found a negative correlation between N added treatments and abundance of N-fixing species, in accordance with other studies (DiTommaso and Aarssen, 1989; Suding et al., 2005; Silvertown et al., 2006).

Although species richness may be a useful indicator of ecosystem functioning (Tilman et al., 1997; Hector et al., 1999; Hector and Bagchi, 2007), in our study CWM but not species richness was correlated with productivity, an aggregate property reflecting ecosystem functioning. In line with the 'mass ratio hypothesis' (Grime, 1998), we found that graminoids, an abundant growth-form in our grassland, contributed significantly to aboveground biomass. However, less abundant traits such as prostrate growth-forms, were also positively related to productivity, suggesting that even the loss of low abundance traits may be of great importance to ecosystem functioning (McLaren and Turkington 2010). Thus, global change drivers that have a significant impact on ecosystem species composition (Dornelas et al., 2014) will most probably have a strong effect on ecosystem processes (Tilman et al., 1997). Beyond functional structure, functional diversity has also been found to be associated to ecosystem processes (Tilman et al, 1997; Valencia et al., 2015). In more productive systems, a few abundant species with similar traits (low functional diversity) usually dominate communities, whereas in low productive systems there is frequently a higher functional diversity (niche complementarity) (e.g. Tilman et al., 1997; Rolo et al., 2016). However, in our grassland we found no relation between functional diversity and productivity, suggesting that under high environmental gradients (precipitation variability and nutrient availability), community structure was the main determinant of productivity as observed for other grasslands (Zhu et al., 2016).

5.6 Conclusions

Our results revealed an important shift in the mean community functional trait composition of our Mediterranean grassland, with decreased water and nutrient co-limitation. Precipitation was the main driver of functional diversity, affecting most of the studied traits. Nutrient addition only decreased growth-form FDis during the very dry year, highlighting the importance of the interacting effects of global change drivers on grassland functional diversity. These results will have strong implication to Mediterranean grassland dynamics mainly within global change contexts. Indeed, higher fertilization and increased drought frequency are likely to affect grassland functional diversity. We show that interacting global change drivers may further constraint trait variability in Mediterranean grasslands. Moreover, higher drought frequency will likely favor graminoids, ultimately affecting the diversity of the seed bank which may decrease, resulting in lower resilience and resistance of these ecosystems to global change (Volaire et al., 2014). Our work further supports that species functional traits may be more predictive of ecosystem processes than species richness making it an important tool to understand ecosystem responses to global changes.

5.7 References

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CHAPTER 6

Synthesis and general discussion

6 Synthesis and general discussion

Climate change models forecast an overall increase in the frequency and intensity of dry spells interspersed with increased occurrence of extreme precipitation events (Miranda et al., 2002; Kovats et al., 2014). Increased use of fertilizers, specifically nitrogen (N) and phosphorus (P), and inputs of reactive N from fossil fuel combustion are other global change drivers increasing nutrient availability in the biosphere (e.g. Peñuelas et al., 2013). Precipitation variability and overall drier conditions as well as nutrient addition are expected to induce great changes in grassland species, community composition, functional structure, and diversity which may affect ecosystem functioning and ecosystem service provision. It is thus crucial to understand and predict the consequences of climate change and nutrient addition for Mediterranean grassland ecosystems.

6.1 Grassland community responses to extended autumn drought and N deposition

Our greenhouse experiment contributed to advance the knowledge on how N deposition interacts with the predicted increase in autumn drought to affect plant species diversity, functional group proportion, productivity and phenology (Chapter 2) as well as CO_2 and water fluxes (Chapter 3). In accordance with results from our field experiment (Chapter 4), we found that N addition did not have a positive effect on aboveground biomass, nor did it affect community species richness or functional group proportion, as our community was co-limited by multiple nutrients. Indeed, although rates of nutrient addition including N was substantially higher in the field experiment (100 kg ha^{-1} yr^{-1}) as compared to the one used in the greenhouse experiment (12 kg ha^{-1} yr^{-1}) and caveat differences in water availability, N did not exert an effect on productivity, as our grassland was also co-limited by other nutrients. Extending autumn drought however, was the main determinant of grassland community responses. We found that a severe drought (i.e. postponing first rains 100 days) decreased productivity, reflecting lower accumulated growing-degree days. On the other hand, communities subjected to a moderate drought (50 days delay) showed a high recovery, as aboveground biomass increased to similar levels as those of the no drought treatment. Nonetheless, in April, these communities had a significantly lower aboveground biomass compared to the no drought, suggesting that if as predicted in autumn and spring

droughts are to be concurrent and more frequent, communities might not recover. Although communities in the severe drought treatment had lower aboveground biomass at the end of the growing season, there was a substantial increment in aboveground biomass in spring, i.e. from April to May. Variations in GPP, probably due to higher leaf-level photosynthetic capacity of younger communities and differences in functional group composition (Chapter 2 and 3), were reflected in biomass recovery in the moderate drought treatment and biomass increment in the severe drought. Indeed, we found that early autumn rains favored large forb species (e.g. rosette species), whereas graminoid proportion was highest in the moderate drought treatment. As legumes usually have high-temperature dormancy, even when water is available (Del Pozo and Aronson, 1999), legumes increased with watering delay, as temperatures at the beginning of the experiment were high compared to temperatures latter in autumn and winter. Functional group response to delayed autumn precipitation was in accordance with results from our field experiment (Chapter 4). Contrary to what would be expected, extended autumn drought did not have a negative effect on species richness. In fact, species richness and diversity were lowest in controls, due to a high abundance of rosette species that showed a competitive advantage in taking up space (bare soil) after early autumn rains, as observed by Espigares and Peco (1993).

Our results also emphasize the ecological importance of early autumn rain events on community phenology. Lower accumulated growing-degree days in the severe drought treatment led to a lower proportion of individuals with flowers and fruits or seeds than in the no drought treatment (Peñuelas et al., 2004; Miranda et al. 2009). This was particularly protruding for forbs, the dominant functional group in our community, which may have important consequences for species composition in the following year (Dudney et al., 2017). Changes in the timing of phenological phases may affect resource availability for herbivores and lead to a mismatch with pollinators (Gordo and Sanz, 2005). This may have a great impact on seed production with cascading effects on community diversity, structure, productivity and ecosystem service provision such as the grassland´s ability to support livestock herding (Gordo and Sanz, 2005; Liancourt et al., 2012).

Besides changes in productivity, community composition and phenology, a delay in first autumn rains significantly affected ecosystem CO_2 uptake and water fluxes (Xu and Baldocchi, 2004; Ma et al., 2007; Jongen et al., 2011; Aires et al., 2008a; Piayda et al., 2014) (Chapter 3). In Mediterranean grasslands, dominated by annual C3 species, first

autumn rains mark the beginning of the growing season and may determine its length (e.g. Xu and Baldocchi, 2004). Earlier leaf area deployment with increased water availability and adequate temperatures, gave the no drought treatment a development advantage compared to communities in the severe drought treatment which explains differences in GPP throughout most of the growing season and reflected on differences in aboveground biomass (Chapter 2). Indeed, variations in GPP, NEE, and ET throughout the growing season were highly correlated with seasonal differences in LAI (Aires et al; 2008 a,b; Jongen et al., 2011). Ecosystem water use efficiency (eWUE) was negatively affected by extending autumn drought, as younger communities, with a potential higher leaf-level photosynthetic capacity, developed in a context of higher water demand (e.g. Huxman et al., 2004). A decrease in eWUE and total GPP as observed in communities subjected to an extended autumn drought may thus reduce community resilience under the predicted scenario of a prolonged dry season or more frequent dry spells (Jongen et al., 2014). Sums of daily NEE, Reco and GPP decreased with the drought treatments. Interestingly, however, in the severe extended drought treatment, N addition had a positive effect on all three flux parameters, attenuating the negative effects of a severe autumn drought on ecosystem CO_2 fluxes. This was probably due to the fact that first watering in this treatment occurred when soil temperatures and thus rates of N mineralization were low (Jarvis et al., 2007; Eviner and Firestone, 2007). In such conditions addition of N must have stimulated CO_2 fluxes as compared to other treatments. Also, as these communities had a high growth increment when temperatures were overall higher these communities must have beneficiated of N supply (Dong et al., 2001; Eviner and Firestone, 2007.

6.2 Grassland responses to number of added nutrients and precipitation variability

Results from our 5-year (2013-2017), manipulative nutrient addition field experiment, clearly demonstrate that productivity of our Mediterranean grassland was co-limited by multiple nutrients and water (Chapter 4) which may help to explain lack of community response to N addition in Chapter two, although N was added at very different rates. Addition of three nutrients consistently (except in 2015, disturbance year) increased productivity compared to controls and one added nutrient treatments. Also, as would be expected for Mediterranean climate regions, water availability was an important factor limiting grassland productivity (Churkina and Running, 1998). Indeed, co-limitation by water and nutrients has also been observed in other Mediterranean grasslands and in

fertilization experiments in arid, semi-arid, and sub-humid regions (Hooper and Jonhson, 1999; Zavaleta et al., 2003; Dukes et al., 2005; Harpole et al., 2007). Also coarse textured soils, such as ours, may further amplify sensitivity to reductions in precipitation (Noy-Meir, 1973).

Moreover, human-induced input of N to ecosystems is widespread and increasing at a higher rate than that of P or other nutrients (Peñuelas et al., 2012; 2013). As grassland communities are nutrient co-limited, the increasing imbalances of nutrients, such as of N:P, confers an increasingly important role to P and other nutrients availability (Vitousek et al., 2010; Ochoa-Hueso et al., 2011; Peñuelas et al., 2012; 2013; Fay et al., 2015).

Even during the drought year (2017; the second hottest and the third driest since 1931) (IPMA, 2017), three added nutrients still exerted a positive effect on productivity as compared to controls, probably due to an increase in graminoid biomass proportion. Our study revealed that the increase in productivity with increasing nutrient addition was mainly attributed to this functional group as was recently also observed in a meta-analysis covering mostly grassland systems in North America and a few studies spread throughout Europe, China, and Australia (DeMalach et al., 2017a). Overall, more added nutrients led to an increase in graminoid and to a decrease in forb proportion (Zavaleta et al., 2003; Harpole et al., 2007; DeMalach et al., 2017a). However, nutrient enrichment interacted with environmental conditions to determine the proportion of these two functional groups. It would be expected that less fertile communities dominated by species with more conservative water use strategies (e.g. species with slower growth rates, such as most forbs) would have a competitive advantage when confronted with drought stress (Grime et al., 2000). However, in our study we found that in the very dry year, in control plots, graminoids increased approximately 37% (compared to previous years) and reached a similar proportion to that of forbs (45% and 55% respectively) and in three added nutrients treatment, graminoids represented 90% of total biomass. In fact this was reflected by the negative response of graminoids to full growing season SPEI. Contrarily, we found that increased precipitation and low evaporative demand in winter exerted a positive effect on forb biomass proportion irrespective of nutrient availability. This competitive advantage of forb species with high precipitation at the beginning of the growing season agrees with the results from our controlled pot experiment (Chapter 2).

The competitive advantage of graminoids in the presence of increased nutrient addition reflects not only their belowground competitive advantage (e.g. greater increase in root cation exchange capacity, higher root density) (Woodward et al., 1984; DiTommaso and Aarssen, 1989; Craine et al., 2001) but also their stronger competitive ability for aboveground resources, such as light (e.g. higher maximum height) (Ansquer et al., 2009; Craine and Dybzinski, 2013). Indeed, we found that increased nutrient addition increased the mean average height of our grassland community, i.e. nutrient addition favored species with a higher maximum height (Chapter 5). Also, during the drought year, three added nutrients increased average community specific leaf area (SLA). This increase in species height and SLA resulted from the increase of graminoid abundance, which were taller and had higher SLA than rosette and erect growth-forms. High SLA, in particular, rendered graminoids better growth performance under drought, which is likely related to their strategy to allocate resources to belowground (Wellstein et al., 2017).

We also found that although legume proportion was unaffected by number of added nutrients, presence of N in treatments had a negative effect on legumes as observed in other studies (DiTommaso and Aarssen, 1989; Suding et al., 2005; van den Berge et al., 2010). In accordance with our results from Chapter two, we found that high precipitation early in the growing season, combined with average lower temperatures, affected positively legume biomass proportion. Consistent with previous work (e.g. Caldeira et al., 2001; Daryanto et al., 2015), legumes where negatively affected by drought. Their lower SLA as compared to that of graminoids could have contributed to their disadvantage during a very dry year. Also, overall less abundant species can be more sensitive to inter-annual climate variability than more abundant species (Cleland et al., 2013).

In chapter five we used a functional trait approach to understand grassland responses to nutrient addition and precipitation variability. As discussed above traits such as SLA, height or growth-form provided a mechanistic understanding of grassland dynamics and responses to environmental conditions. We found that nutrient addition, precipitation and in some cases their interaction induced significant changes in community functional trait structure (CWM), with addition of three nutrients and a very dry year (2017) leading to the greatest shifts (Chapter 5). For instance, changes in community SLA only responded to the addition of three nutrients. This was also observed in a study based on data from 27 NutNet sites (our grassland included) (appendix 1). Indeed, the effectiveness of a change in community structure only with the addition of three nutrients is in line with community

co-limitation by multiple nutrients (Chapter 4). More importantly, the study revealed that although SLA is a commonly used leaf trait to evaluate the functional responses of plant communities to environmental changes it also presented the greatest variation due to abiotic and biotic conditions as compared to other traits such as nutrient content.

Along with changes in functional group proportion, increasing number of added nutrients led to a decrease in species richness (Chapter 4). The niche dimensionality hypothesis, predicts that higher diversity of co-existing species in a community results from higher niche dimensionality (Hutchinson, 1957), i.e. species diversity is maintained by functional trade-offs among species for below and aboveground resources (Tilman, 1982). In an attempt to test the niche dimension hypothesis, Harpole and Tilman (2007) found that species richness decreased with decreasing nutrient and water limitation. In our study we also found that addition of three nutrients had consistently, throughout our five year study, a negative effect on grassland species richness, in line with the niche dimensionality hypothesis (Chapter 4). As nutrient addition increases plant biomass, light limitation (e.g. Tilman, 1987; Goldberg and Miller, 1990; Hautier et al., 2009) or more specifically an increase in the size asymmetry of light partitioning vertically throughout the canopy (DeMalach et al., 2017b) has been found to be the main mechanism behind the negative effect of nutrient enrichment on species richness. Taller and larger species at maturity receive more light per unit biomass than smaller plants, causing competitive exclusion and thus reducing species diversity (Tilman, 1987; DeMalach et al., 2017b). Indeed, we found that increased nutrient addition favored species with a higher maximum height (Chapter 5). Addition of multiple nutrients can shift competition from below-ground resources to above-ground resources i.e. for light (Harpole et al., 2016). Interestingly, however, although community average height also increased with precipitation, this did not induce species loss, suggesting that a combination of above- and below-ground competition might be the cause of competitive exclusion and thus reduced species richness as observed by Rajaniemi (2002).

Contrary to the observations of Harpole and Tilman (2007) and DeMalach et al. (2017a) decreased water limitation (i.e. normal and wet precipitation years) and nutrient addition did not increase species loss. In fact, it was during a drought year (2017), that species richness decreased the most in all nutrient treatments, suggesting that droughts are an important driver of plant species richness in Mediterranean grasslands irrespective of nutrient availability. This was also recently observed by Copeland et al. (2016) for annual

plant communities in both infertile serpentine and fertile non-serpentine soils in California when subjected to an extreme drought.

Besides the negative effect on current-year species richness, droughts have also been found to preclude species richness recovery, even though normal precipitation years may follow (Tilman and El Haddi, 1992). Indeed, previous-year precipitation, i.e. lagged effects created through seed production and litter, is a key determinant in structuring annual community composition and diversity (Dudney et al., 2017). In this line of reasoning, the decrease in species richness in 2017 was probably not only the outcome of current year low precipitation coupled to higher spring temperature and increased evaporative demand, but also a result of previous year low precipitation and a trend towards decreased richness in 2015 and 2016. This suggests that if as predicted, droughts are to be more frequent, this may constraint species richness and trait variability in Mediterranean grasslands which has been found to be essential to sustain ecosystem processes (Hector et al., 1999; Caldeira et al., 2005; Hector and Bagchi, 2007). Indeed, we found that precipitation was the main driver of changes in functional structure and diversity (FDis) (Chapter 5). For most of the studied traits, functional diversity decreased during a drought year, as was the case for vegetative traits, dispersal mode and nutrient uptake. On the other hand, functional diversity of reproductive traits (onset of flowering and flowering duration) increased during the drought year. This co-existence of species with contrasting reproductive strategies, may assert not only a certain degree of resilience to drought, but also sustain ecosystem functioning as would be expected from niche complementarity (Mason et al., 2005; Díaz et al., 2007; Valencia-Gómez et al., 2015). Different traits showed divergent responses to environmental variation which may suggest trade-offs among species strategies (Bernard-Verdier et al., 2012; Carmona et al., 2015; Rota et al., 2017). Single-trait analyses capturing different functional niches revealed differences in community response patterns to precipitation that otherwise might have been overseen if we had condensed trait response into a single index (Bernard-Verdier et al., 2012; Butterfield and Suding, 2013). However, in our study most traits that responded to precipitation showed a negative trend in FDis with increasing drought. Therefore, in our case, the use of a multi-trait was useful to summarize FDis grassland response to drought.

Co-existing species, which make up Mediterranean grasslands, represent different evolutionary adaptations to cope with water limitation and nutrient constraints. (e.g. Noy-Meir, 1973; Peco et al., 1998; Pérez-Camacho et al., 2012). Indeed, the variability in trait

functional structure and diversity that we found throughout our four contrasting climatological years and the pronounced differences in trait functional structure along a gradient of nutrient availability, shows clearly the alternative evolutionary “solutions” that species have developed to deal with multiple and complex situations. It is this “potential” structural and functional diversity which contributes not only to sustain grassland functioning but also to sustain grassland resilience to different constraints (e.g. drought) (e.g. Volaire et al., 2014; de la Riva et al., 2017). Nonetheless, if spring and autumn droughts are predicted to increase in intensity and frequency (Kovats et al., 2014), the decrease in functional diversity and changes in community structure that we observed may have lagged effects and weaken community resilience.

In the third year of our field study (2015), heavy defoliation by cattle combined with winter and spring drought, critically affected grassland productivity and functional group composition (Chapter 4). In particular, treatments with more added nutrients recorded the highest decrease in productivity in 2015 compared to other years. Whereas, for controls, productivity in 2015 was only lower than that of the highest productivity year. Our results showed that grasslands with higher nutrient availability were less resistant to the disturbance event. Reduced resistance was associated to high canopy development of three added nutrient communities early in the growing season, as confirmed by leaf area index measurements, and most probably higher palatability, which made these communities be preferred by cattle (Rosenzweig, 1971; Mattson, 1980; Knapp et al., 2012; Peco et al., 2012). Also, in line with the diversity-stability hypothesis (Yachi and Loreau, 1999; Caldeira et al., 2005; Tilman et al., 2014), lower species richness contributed to lower resistance of these communities. Nutrient enrichment and dominance of a competitive functional group such as graminoids with higher growth rates contributed to increased recovery as species were able to take advantage of increased resource availability the next year after the perturbation, compared to species that dominated controls and one added nutrient treatments. Antagonistic responses of resistance and recovery to the 2015 disturbance determined no differences in resilience between communities subjected to different nutrient treatments, in accordance with productivity stability results. Indeed, although three added nutrients led consistently to a decrease in species richness, this did not affect productivity stability (5-year data) as would be expected from most biodiversity–ecosystem function studies based on controlled experiments (e.g. Tilman et al., 1997; Caldeira et al., 2005). When resources, including nutrient availability, differ between communities this relationship may not hold (Grman et

al., 2010; Hautier et al., 2014; Xu et al., 2015). Species asynchrony (Hautier et al., 2014) or dominance of particularly stable species in fertilized plots can contribute to maintain stability in spite of decreased species richness (Grman et al., 2010).

Species richness is a useful predictor of ecosystem functioning (Tilman et al., 1997; Hector et al., 1999; Hector and Bagchi, 2007). Functional traits, however, may provide a more mechanistic understanding of species functional role in ecosystems and help to predict community response to environmental conditions (e.g. Petchey and Gaston, 2006, Cadotte et al., 2011). Indeed, we found that functional traits, related to competitive strategies for resources (e.g. light) or to resource-use strategies, were better related to productivity than species richness. Increased height was positively related to productivity in accordance with a higher community height in more productive communities. In line with the mass ratio hypothesis (Grime, 1998), we found that graminoids (an abundant growth-form) was positively related to productivity, whereas rosette growth-forms (e.g. some forbs) were negative related to productivity (Chapter 5), in agreement with results from Chapter 4. Although dominant traits tended to have the greatest influence on communities, less abundant traits (e.g. prostrate growth-forms) also influenced productivity, suggesting that even the loss of low abundance traits may be of great importance to ecosystem functioning (e.g. light interception, soil moisture and soil nutrients) (McLaren and Turkington, 2010) or for instance invasion resistance (Lyons and Schwartz 2001). Besides the positive effect on productivity, the observed increase in the abundance of graminoids, species with overall higher growth and nutrient acquisition rates, with nutrient addition and increased drought, may likely affect other ecosystem processes (e.g. litter decomposition, nutrient cycling, water storage) (Hooper and Vitousek, 1997; Garnier et al., 2004; Harpole at al., 2007). Indeed, species with higher SLA have low tissue density, lower lignin content and thus higher decomposition rates (Lavorel and Garnier, 2002; Garnier et al., 2004). Higher growth rates may in turn lead to high rates of CO_2 sequestration, nutrient uptake and water demand (Lavorel and Garnier, 2002; Harpole et al., 2007) which may be critical under the predicted increasing frequency of droughts for the Mediterranean region.

6.3 Future Challenges

This thesis contributed to better understand the fundamental role of seasonal autumn precipitation in modeling grassland community composition, diversity, phenology,

productivity and carbon and water fluxes of Mediterranean grasslands. Our work contributed to understand how a Mediterranean grassland responded in terms of plant species richness, productivity and community composition to decreased nutrient limitation and precipitation variability. This study revealed consequences of nutrient enrichment on grassland resistance and recovery to disturbance. We disclosed important shifts in community functional structure due to nutrient addition and precipitation, and pointed out to the importance of precipitation as a major determinant of community functional diversity, highlighting the importance of droughts in modeling Mediterranean grassland ecosystems. Our work further supports that species functional traits may be more predictive of ecosystem processes than species richness making it an important tool to understand ecosystem responses to global changes.

Future research should encompass long term studies in order to comprise as much of the climatological fluctuation characteristic of the Mediterranean climate. As higher frequency of droughts will induce new species dominance patterns, this will ultimately affect seed bank diversity. The study of their composition and viability may further help to understand grassland resilience to frequent droughts and help to define management options. Additionally, future studies should include intraspecific trait variability to strengthen the predictions of climate change impacts and nutrient addition on annual grasslands. This may be particularly important for traits such as SLA which have high intraspecific variability as was acknowledge in the collaborative manuscript (appendix 1).

Future research should also address the possibility of upscaling information from local plant sampling to regional and broader spatial scales using remote sensing information. Indeed, as shown in appendix 2, the use of specific wavebands which represent different traits expected to change throughout the growing season (e.g. vegetation water content) in conjunction with vegetation indices may substantially improve GPP estimates. This may be particularly important for monitoring and modelling GPP of grasslands under the predicted increase in precipitation variability by providing early signals of disturbance and thus help to develop adequate management options.

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APPENDIX Collaborative Work

Appendix 1

Leaf nutrient contents but not specific leaf area increase rapidly and predictably in response to eutrophication.

Submited to Nature Ecology & Evolution on the 3rd of June 2018. In review:

Collaborative authorship

Authors	Developed and framed research question(s)	Analyzed data	Contributed to data analyses	Wrote the paper	Contributed to paper writing	Site coordinator
J. Firn	x	x		x		x
J. McGree	x	x		x		
E. Harvey	x	x			x	
J. W. Morgan					x	x
N. Eisenhauer					x	x
P. A. Fay					x	
S. Prober	x				x	x
K. J. La Pierre					x	x
C. A. Arnillas					x	x
L.. Biederman					x	x
W. S. Harpole					x	x
A. C. Risch	x		x		x	x
M. Schütz	x		x		x	x
R. Mitchell					x	x
J.Wright					x	x
C. Nogueira					x	x
E. Seabloom					x	x
P. D. Wragg					x	x
C. Roscher					x	x
M. N. Bugalho					x	x
N. Hagenah					x	x
E. Cleland					x	x
J. L. Moore					x	x
M. Smith					x	x
C. S. Brown					x	x
Y. M. Buckley	x				x	x
Y. Hautier					x	x
P. L.Peri					x	x
C.Stevens					x	x
E. T. Borer					x	x

P. B. Adler	x	x
A. R. Kleinhesselink	x	x
E. Ladouceur	x	
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A. A.D. Broadbent	x	
A. Ebeling	x	x
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Abstract

Theory predicts that plant functional traits rather than species composition provide a common currency for understanding how anthropogenic pressures such as eutrophication and reduced herbivory impact the functioning of grasslands. Here, we show for the first time in a global experimental network comprised of 27 grassland sites across fourty countries that one of the most commonly measured leaf traits, specific leaf area (SLA, a composite measure of leaf area per unit mass), does not increase significantly in response to fertilizer and herbivore exclusion treatments as theory predicts. Leaf nitrogen, phosphorus and potassium contents did increase in response to soil nutrient addition, but contrary to expectations, we found few significant increases when vertebrate consumers were excluded. We also found significant context dependency in how leaf traits changed depending on species turnover over time in response to treatments and climatic and soil nutrient conditions. We revealed generalizable local response syndromes (explained by combinations of intraspecific and interspecific trait variation) where plants change physiologically without necessarily investing differently in leaf area, suggesting that leaf traits such as SLA may not be appropriate indicators of adaptation to short-term perturbations.

The irreplaceable loss of biodiversity is accelerating at an alarming rate globally, with local biodiversity losses already predicted to be beyond safe limits (Barnosky et al., 2011), particularly in grasslands (Newbold et al., 2016). Two of the most significant threats to biodiversity are eutrophication with increasing agricultural practice and industrial pollution (Rockstrom et al., 2009) and changed trophic level interactions such as reduced consumption by hervivores (Estes et al., 2011; Ripple et al., 2015). These two drivers also impact species persistence, potentially selecting for some traits and not others, and thereby affecting ecosystem functioning (Borer et al., 2004). Functionally relevant traits, rather than species richness, have been increasingly used as a "common currency" to assess the consequences of biodiversity loss (Shipley et al., 2006; Funk et al., 2017) on ecosystem functioning (Hooper et al., 2005; Messier et al., 2010).

Leaf traits are commonly used to predict plant-animal interactions and plant species responses to environmental changes (Suding et al., 2008). Plants invest photosynthate and mineral nutrients in the construction of leaves, which capture light to produce more photosynthate (Wright et al., 2004, 2005). Commonly measured leaf traits, such as leaf nutrient content and specific leaf area (SLA), are commonly used as comparative measures of how plants capitalize on their investments. SLA is measured as leaf area per unit mass and represents a tradeoff between surface area for capturing photons and thickness related to structural adaptations for water conservation and herbivore defence. Indeed, leaf traits correlate across a continuum of fast to slow returns-on-investment, known as the leaf economic spectrum (LES) (Díaz et al., 2004; Wright et al., 2004; Westoby and Wright, 2006). Fast-growing species, which are adept at resource acquisition and tend to dominate more productive climates and soils where resource availability is not limiting, have high SLA and leaf nutrient contents. High SLA is associated with lower costs of leaf construction, higher species turnover rates in plant communities and higher rates of herbivory as tissue becomes more palatable. Additionally, higher species turnover and palatability are also positively correlated with leaf nitrogen (N), phosphorus (P), and potassium (K) contents (Díaz et al., 2004; Wright et al., 2004; Westoby and Wright, 2006).

By contrast, slower-growing species, which exhibit resource conservation, are characterized by low SLA and leaf nutrient contents (Díaz et al., 2004; Wright et al., 2004; Westoby and Wright, 2006). As a result, slow-growing species are less palatable to herbivores, while having a longer leaf life span. These trade-offs in leaf traits across the LES emerge over evolutionary time-spans as species adapt to particular nutrient and

herbivory conditions. However, the extent to which rapid changes in these structuring forces result in parallel shifts in trait values of plant communities is not known (Funk et al., 2017). This is especially because a long-held hypothesis in agriculture postulates that leaf nutrient content may not increase in response to nutrient addition because increased plant growth can outpace nutrient accumulation in tissue, which is known as the dilution effect (Jarrell and Beverly, 1981).

Based on resource acquisition-conservation tradeoffs, SLA and leaf nutrient contents are commonly used as indicators of plant-level responses to environmental changes (Suding et al., 2008). However, leaf trait responses of individual species are not only influenced by wide-scale environmental change but are also dependent on local-scale abiotic and biotic processes. Temperature and precipitation gradients and variations in soil characteristics can occur at broad spatial scales (Ordonez et al., 2008). Local climatic and geological variation interact with drought, fertilization, changes in natural disturbance regimes and invasions of non-native flora and fauna (Lavorel and Garnier, 2002; Leishman et al., 2007; McIntyre, 2008; Dwyer et al., 2014; Firn et al., 2017) to sculpt ecosystem structure and function. Given the complex sets of interactions that may explain leaf trait responses to short-term changes in environmental conditions, a modelling approach that evaluates these potentially interacting factors is necessary to discern underlying processes that may otherwise be missed when using traditional bi-variate approaches (Grace et al., 2012; 2016).

In this study, we quantified how leaf traits of the most dominant species in grasslands change in response to the addition of soil nutrients (i.e., N, P and K) and the exclusion of vertebrate herbivores. We compiled leaf traits from the Nutrient Network (NutNet) (Borer et al., 2014) cross-continental distributed experiment established at 27 grassland sites (Fig. 1, Table S1). This experimental network allowed us to test how commonly measured leaf traits respond to environmental change across grassland sites characterized by a diverse range of climatic and edaphic conditions that were subjected to identical experimental treatments.

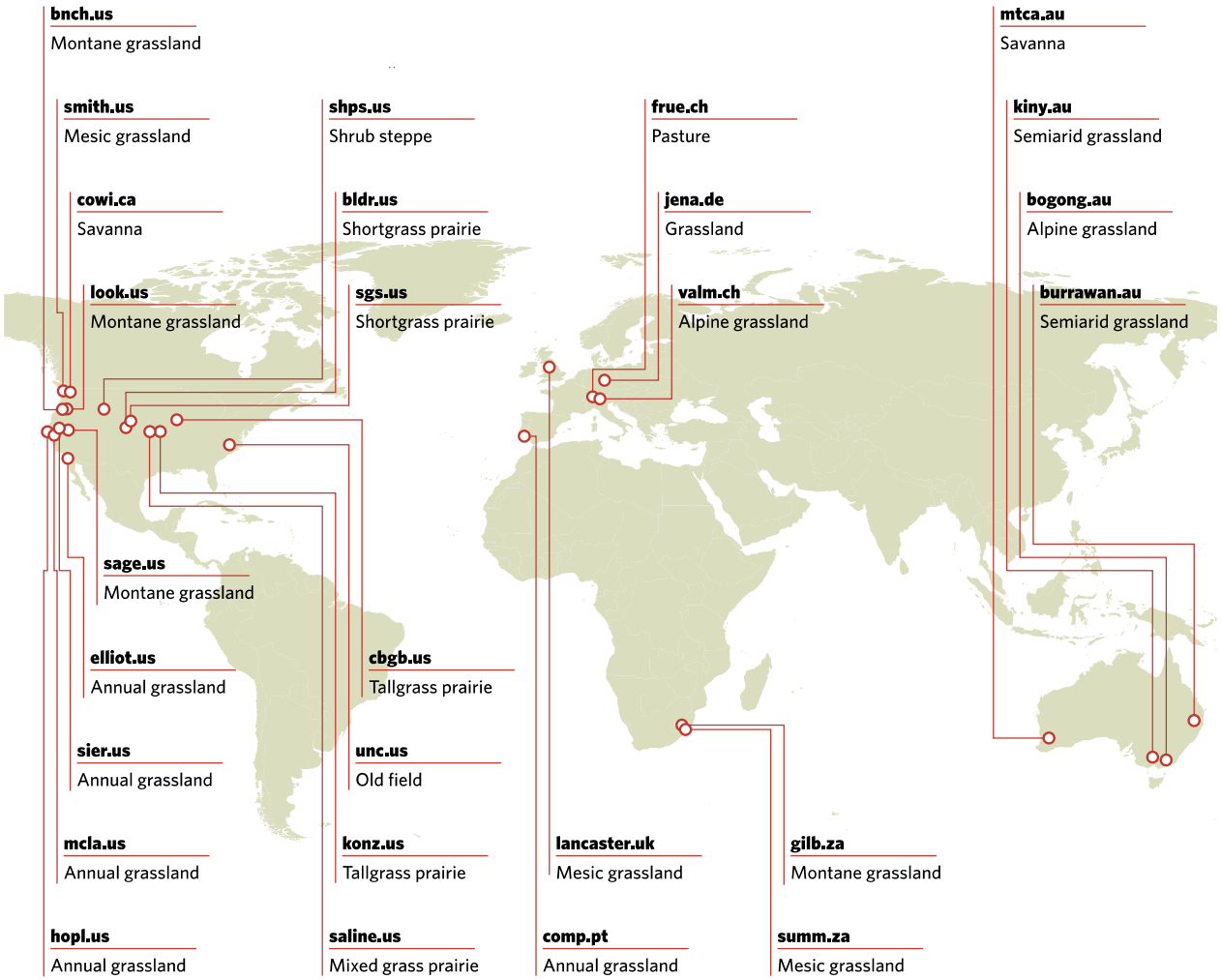

Fig. 1 Map showing the locations of the 27 Nutrient Network study sites across four continents and eight countries where traits leaf traits were collected from each experimental treatment using a standardized protocol. au = Australia, ca = Canada, ch = Switzerland, de = Germany, pt = Portugal, uk= United Kingdom, us = United States, za =South Africa.

At each NutNet site, three blocks of ten 5- x 5-m plots were established, and then two experiments were initiated: 1) a full factorial nutrient addition experiment, including the addition of all factorial combinations of N, P and K+µ, which included ten other micronutrients (see Borer et al. 2014 and Methods for more detail) and 2) a factorial nutrient addition (NPK+µ addition) by vertebrate herbivore exclusion experiment (>50 g; details in Methods summary and Supplemental Materials). We collected leaves from five individuals of the most dominant species in each of the experimental treatments and replicate plots (see Methods). Overall, 243 species were sampled across the 27 sites,

including grasses, forbs and legumes, and 2664 leaf samples (comprised of several leaves per species per plot) were measured for leaf area, leaf dry weight, and leaf N, P and K contents (Cornelissen et al., 2003) . Leaf traits were collected from the most dominant species whose cover represented 26% of the total vegetation cover. Cover values ranged from 5% at the highest elevation (Val Müstair, Switzerland), which had the highest average species richness per plot (25 species in 1-m by 1-m survey plots), to 52% in a mixed grass prairie in the central plains of the USA (Saline USA) that is dominated by two species. The effect sizes of the mean trait values for all species in response to the experimental treatments were estimated using multilevel regression models in a hierarchical Bayesian framework with integrated nested Laplace approximation (Rue et al., 2009).

N, P and K leaf contents increased significantly and in tandem when these nutrients were added to the soil (Fig. 2). Leaf N also increased when P and K were added to the soil, and the leaf P content decreased marginally in treatments that received only N (i.e., where P was not added; Fig. 2c), suggesting that soils are likely limited by both N and P and providing evidence of nutrient co-limitation (Bracken et al., 2015). The leaf K content showed the highest variation when compared to the other leaf traits and depended on species (~60%, Fig. 3), which suggests the capacity to take up more K when soil supply is elevated is species specific. The exclusion of herbivores alone did not significantly alter the leaf nutrient contents (Fig. 2).

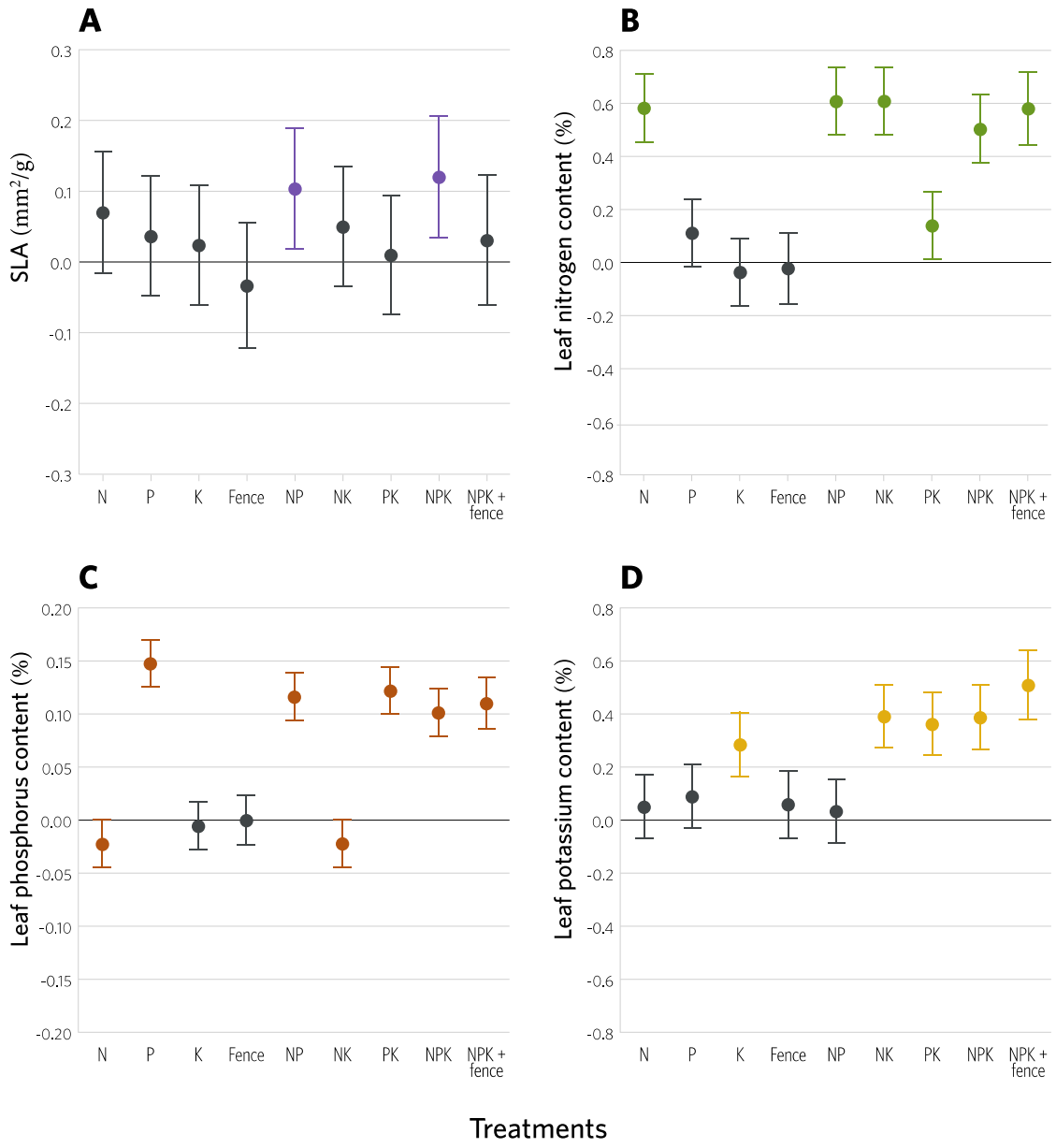

Fig. 2 Comparison of effect estimates: error bars represent 95% credible intervals, and the 0 reference line represents the intercepts of the models that were the control treatment for a) specific leaf area (SLA) (purple coloration indicates significant differences among treatments) b) leaf N content (green coloration indicates significant differences among treatments), c) leaf P content (red coloration indicates significant differences among treatments), and d) leaf K content (yellow coloration indicates significant differences among treatments) from Bayesian hierarchical models fit with INLA with a random effects structure of species/site/block. Intercept values represent leaf trait estimates in the control treatments, and the values are a) SLA = 8.7 ± 0.6, b) leaf N content = 2.7 ± 0.3, c) leaf P content = 0.2 ± 0.04, and d) leaf K content = 1.9 533 ± 0.30.

Although SLA is commonly used to evaluate the functional responses of plant communities to environmental changes (Funk et al., 2017), we did not find a strong and consistent increase across grassland sites in response to nutrient addition or vertebrate herbivore exclusion (Fig. 2a). We found a small but significant increase in SLA in the NP (mean SLA = 8.79 mm2/g) and NPK fertilizer treatments (mean SLA = 8.81 mm2/g) compared to the control (mean SLA = 8.69 mm2/g), suggesting a requirement for both N and P to increase SLA (Fig. 2a) (Elser et al., 2007). SLA showed the highest variability of any of the measured leaf traits at the site level (Fig. 3 ~75% of the variation in SLA in response to treatments was explained among sites), which suggests that SLA variation may be explained by local abiotic and biotic conditions.

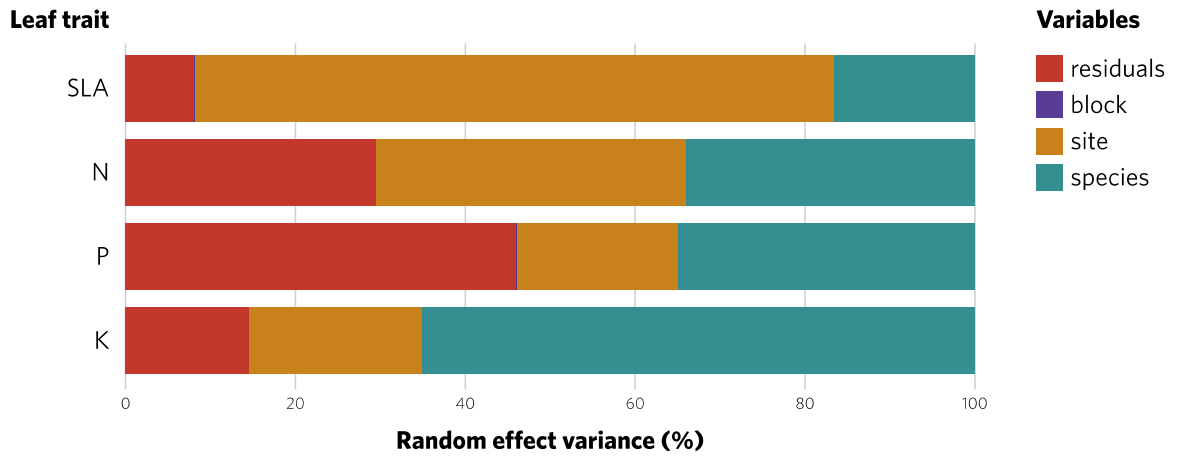

Fig. 3: Percentage of variation explained by the random effects of block nested in site nested in species including residual variation from the Bayesian hierarchical models 540 fit with INLA for each of the four leaf traits (response effects are shown in Fig. 2) including specific leaf area (SLA), leaf nitrogen content (N), leaf phosphorus content (P), and leaf potassium content (K).

To investigate correlations among leaf traits, including all possible pairwise combinations, both overall and grouped by experimental treatments, we used standardized major axis regression (Warton et al., 2011). We found positive correlations between all pairwise leaf nutrient content relationships (Fig. S1), and pairwise relationships between leaf nutrients showed expected increases or decreases in slope elevation in response to nutrient addition. However, positive correlations between SLA and leaf nutrient contents were

weak overall and not significant when grouped by experimental treatments (i.e., SLA/leaf nutrient content relationships were not significant; Table S2-S6).

The increased leaf nutrient contents can be explained by intraspecific trait variation (increases shown by the same species over time) or by interspecific changes in dominant species following the application of treatments. Three years after treatment initiation, changes in dominant species were observed at some study sites, whereas little change was observed at other sites. Mechanistically, this difference is important as it helps us to further understand whether the changes measured occurred because dominant species were able to take up additional nutrients or whether new species were recruited, and consequently, the increased nutrient contents in the leaves can be explained (Albert et al., 2010). Therefore, we evaluated the effects of temporal species turnover on leaf trait responses. To do this, we estimated temporal species turnover using Bray Curtis dissimilarity for the most dominant species in each plot depending on species composition at time 0 and again when the leaf traits were measured, usually after three years.

Given the global extent of our study sites and the high amounts of variation in leaf traits found at the site level, particularly for SLA (Fig. 3), we also evaluated the effects of climatic conditions and pre-treatment soil nutrient levels using structural equation models to examine the influence of these additional possible intrinsic and extrinsic drivers and to conduct system-level analyses (see supplementary material for details on model development). Overall, the R^2 values for each of the leaf trait response variables were high, indicating a strong explanatory power of the models; leaf K had the highest R^2 value and SLA the lowest (leaf N, R^2 = 0.53; leaf P, R^2 153 = 0.32; leaf K, R^2 = 0.55; and SLA, R^2 154 = 0.11).

Climatic and edaphic conditions significantly explained the variation in all leaf traits (Fig. 4 and Fig. S6). The nutrient addition treatments explained considerable amounts of variation in the leaf nutrient contents but not in SLA. Species temporal turnover was positively correlated with leaf nitrogen and potassium contents, but significant correlations were not found with the leaf phosphorus content or SLA. This result suggests that a portion of the increase in the leaf nitrogen an potassium contents was explained by interspecific variation, suggesting a selection effect of the addition of these nutrients on species composition, whereas the positive response of leaf phosphorus was largely explained by intraspecific trait variation. The duration of the nutrient addition treatments was also

positively correlated with species temporal turnover, which suggests that sites that were treated for longer had higher species turnover. Co-variances among the leaf nutrient contents were high in the structural equation model, but SLA again showed the lowest co-variation with the leaf nutrient contents (Table S7).

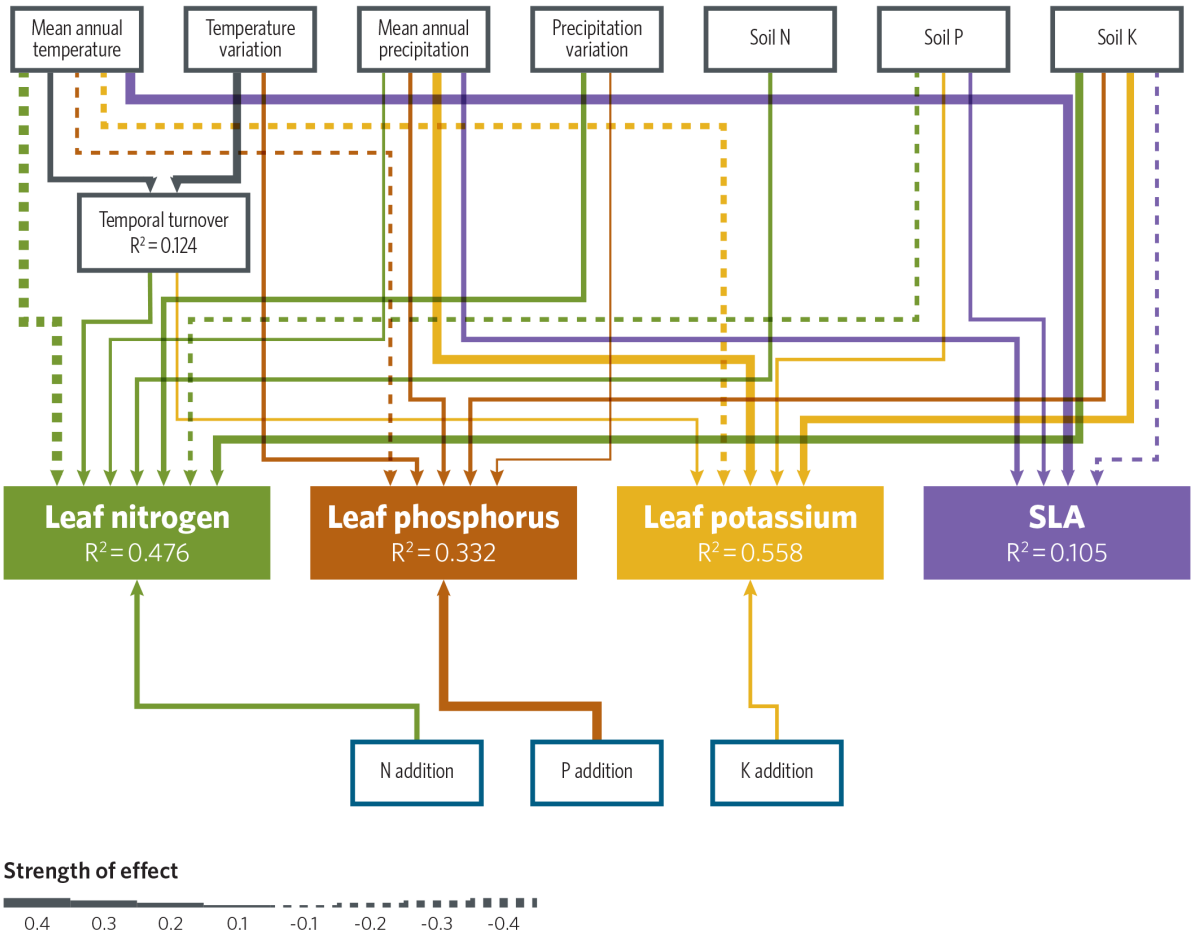

Fig. 4 Structural equation model diagram representing connections between leaf traits, experimental nutrient addition treatments, site-level average climatic and pre-treatment edaphic conditions, as well as species turnover. The width of the connections represents estimates of the standardized path coefficients, with solid lines representing a positive relationship and dashed lines a negative relationship. Only significant connections are shown; path coefficients are presented in Fig. S6 of the supplementary material.

Before trait-based ecological studies can scale the responses of leaf traits from individuals to communities and ecosystems (lavorel and Garnier, 2002), a more definitive understanding of when, where and how to interpret changes in plant trait values is needed.

This includes how to match different plant expressions to appropriate environmental conditions depending on the characteristics of specific ecosystems. This necessitates testing plant trait responses in experimental studies, particularly in relation to local and short-term environmental changes or disturbances (Funk et al., 2017). In general, in the first global common experimental test of the leaf trait responses, the leaf nutrient content responded consistently to short-term nutrient additions, and this response is explained by both changes in species identity in response to the treatments and the ability of the same dominant species to take up more nutrients when available (Firn et al., 2012). We found little evidence of a consistent short-term response of SLA and leaf nutrient contents to reduced vertebrate herbivory. The SLA of the dominant species did not increase consistently in response to short-term treatments, except where both N and P were added to the soil. However, once climatic and edaphic conditions were considered in the analyses, SLA showed no significant response to the treatments.

Our findings have implications for how leaf traits are used to infer responses to local-scale environmental perturbations within grassland ecosystems. SLA should be interpreted carefully when used as a proxy for functional response to environmental change within grasslands. SLA has been found to be a reliable indicator of plant resource utilization strategies at macro-scale gradients of environmental change that sculpt and filter plant traits over longer time frames (Dwyer et al., 2014). However, our first global-scale experimental test demonstrated that leaf morphology is not a consistent indicator of the short-term response of plants to increased soil nutrients. Our findings are akin to the criticisms of the adaptationist programme in evolutionary biology, which challenges the validity of attributing changes in individual traits of organisms in response to abiotic and biotic factors as examples of adaptation (Gould and Lewontin, 1979). Changes in individual traits, in the same species or because of species turnover, do not necessarily represent evidence of adaptation. In addition, the application of broad-scale biogeographical trait relationships across ecosystems, such as the worldwide leaf economic spectrum (Wright et al., 2014), does not necessarily correlate with a short-term adaptive response to disturbance and changing abiotic conditions. In the most dominant species, leaf nutrients are responsive to nutrient additions, even across grasslands characterized by very different climatic and edaphic conditions, and are potentially more consistent plant functional response traits, particularly over short term periods.

Methods summary

Network of experimental sites

The 27 study sites are part of the Nutrient Network, a cooperative globally distributed experiment (Fig. 1 and Table S1 in Supporting Information, http://www.nutnet.org/). Each experimental site had a randomized block design, and at most sites, three replicate blocks divided into ten 5- x 5-m plots were established, resulting in a total of 30 plots per site (Table S1 Supporting Information). We quantified climatic variables for each site using modelled values sourced from the WorldClim Global Climate database (version 1.4; http://www.worldclim.org). The sites included in this study represented a wide range of climatic conditions with mean annual temperatures ranging from 0.3 ºC (alpine grassland in Switzerland) to 18.4 ºC (semi-arid C4 perennial grassland in Australia) and mean annual precipitation ranging from 262 mm (shrub steppe in the USA) to 1898 mm (montane grassland in the USA).

Nutrient addition experiment

In this experiment, we established a set of nutrient addition treatments that included a full factorial combination of three essential plant macronutrients (N, P, K plus micronutrients), including a control. The following rates of nutrients, obtained from the same chemical sources, were applied at all sites: 10 g N m^{-2} yr^{-1} as timed release urea, 10 g P m^{-2} yr^{-1} as triple super phosphate, and 10 g K m^{-2} yr^{-1} as potassium sulphate plus a once-off addition (100 g m^{-2} yr^{-1}) of macro- and micro nutrients (i.e., Fe, S, Mg, Mn, Cu, Zn, B, Mo, Ca). At all sites, N, P, and K fertilizers were applied annually, whereas micro-nutrients were applied once at the start of the study to avoid toxicity and only in treatments that included K. Nutrient treatments began in 2007-2014 across the study sites; nutrients were only applied for two years at two of the sites, compared to three to four years at the remaining sites. Note that ammonium nitrate was used in 2007 at some sites before switching to urea because of increasing difficulty in sourcing ammonium nitrate globally. At a subset of these sites, we tested whether this one-year addition of ammonium nitrate would influence the outcomes of the plant community responses and found no significant effect of nitrogen source (Borer et al., 2014)

To analyse soil nutrients prior to treatment initiation, we first removed the litter and vegetation from the soil surface and then collected two soil cores (2.5 cm in diameter and 10 cm deep) from each plot. The plot subsamples were composited, homogenized, and air-dried. The Ecosystems Analysis Laboratory at the University of Nebraska assayed the soils to determine C (%) and N (%) using dry combustion GC analysis (COSTECH ESC 4010 Elemental Analyzer, Costech Analytical Technologies, Valencia, California, USA). Extractable soil P and K and soil pH were assayed at A&L Analytical Laboratory (Memphis, TN). Soil pH was measured using a 1:1 soil to water slurry.

Nutrient addition by herbivore exclusion experiment

The vertebrate herbivore exclusion treatment was established by fencing two plots within each of the blocks. We designed the fences so that they would effectively exclude large aboveground mammalian herbivores, including ungulates, across a diverse range of grasslands characterized by different herbivores (Borer et al., 2014). At the majority of sites, the height of the fences was 180 cm, and the fence design included wire mesh (1-cm holes) across the first 90 cm in addition to a 30-cm outward-facing flange stapled to the ground to exclude burrowing animals; climbing and subterranean animals could potentially have accessed these plots.

Cover sampling within treatment plots

At peak biomass, species areal cover was visually estimated using a modified Daubenmire method (Daubenmire, 1959), where cover is estimated to the nearest 1% within one 1-m^2 sub-plot in each plot. Cover was estimated independently for each species, so the total summed cover may have exceeded 100% for multilayer canopies. After three years of treatments at most sites, we used the cover estimate data to select the top three to eight species in each subplot to measure leaf traits. We chose to identify the most dominant species in each plot as opposed to at a site-level sampling approach because we wanted to capture the full range of the responses to the treatments. After three years, the different treatments at some sites exhibited a high species turnover, which represents community and ecosystem level responses to treatments.

Leaf trait collection and analyses

Predominantly in 2009, we randomly collected five fully developed leaves with no signs of herbivore damage from five mature individuals of the three to five most dominant species in each plot (although the eight most dominant species were sampled at one site) using the standardized protocols detailed by Cornelissen et al. (2003). All leaves from each species in each plot were combined when scanned to measure leaf area using a flatbed scanner (Epson perfection V300) and image analysis software ImageJ (Abramoff et al., 2004) or various leaf area meters depending on whether a leaf area meter was available to a principle investigator. Thereafter, all leaves were dried at 60 ºC for 48 h and were then weighed (dry weight); SLA was calculated as leaf area (mm^2)/dry weight (g). SLA was calculated for all five leaves collected from each species in each plot and then divided by five to derive a species-level mean for each plot.

Dried leaves were then ground, bulked per plot and per species and analysed for leaf nutrient contents. The leaf nitrogen content was determined using a LECO TruMac, which is based on a combustion technique that uses thermal conductivity relative to pure gas; the leaf nitrogen content is determined and is considered accurate to within 1%. The leaf potassium, and phosphorus contents were determined using laser ablation ICPMS after Duodu et al. (2015) with the following exceptions: the internal standard was not added but was measured C, the most abundant naturally occurring element was used, and no extra pulverizing was performed beyond that required for C and N analysis, which consisted of placing a sample and a 2-mm-diameter tungsten carbide ball inside 2-mm plastic centrifuge vials, followed by grinding for 15 min using a TissueLyser©. Leaves (approximately 0.2 g) were compressed in a hydraulic dye, which produced a pellet approximately 5 mm across and 2 mm tall. These pellets were glued to a plastic tray in groups of ~100 and were placed inside the laser chamber. A New Wave 193-nm excimer laser with a True-line cell was connected to an Agilent 8800 ICPMS. The laser beam was 65 microns in diameter and was rastered across a length of approximately 500 microns for approximately 50 seconds, five times per sample with a 30-second washout or background between rasters. The laser fluence at the laser exit was approximately 2 J/cm^2, and the repetition rate was 7 Hz. The reference material was NIST NBS peach leaves (USA National Institute of Standards and Technology, 2017), and NIST NBS spinach (USA

National Institute of Standards and Technology, 2014) was used as a monitoring standard; these were analysed every three samples (15 rasters) for moderately close sample-standard bracketing. The average and standard deviation of each element in each sample were calculated and reported after the method presented by Longerich et al. (1996) using Iloite data reduction software (Paton et al., 2010).

Data analyses

Hierarchical Bayesian multilevel regression models

We developed multilevel regression models in a hierarchical Bayesian framework. All analyses were run using the integrated nested Laplace approximation (INLA, Rue et al., 2009) interfaced with the R statistical computing package (R Development Core Team, 2013). The default priors in INLA were used for all analyses, which included the normal distribution specified as N (mean, precision), fixed effects: intercept = N (0,0), slopes = N (0,0.001), and variances modelled as log-precision with priors of log-gamma (1, 5e-5), which was specified as log-gamma (shape, inverse-scale).

For each of the overall data (nutrient addition experiment or nutrient addition by herbivore exclusion experiment) for each of the leaf traits (i.e., specific leaf area, leaf N, P and K content), let y_{ijkl} denote the response and $\boldsymbol{x}_{jk} = (x_{1jk}, x_{2jk},\ldots,x_{pjk})$ denote the *i*th observation from the *j*th block at the *k*th site of the *l*th plant species (Fig. M1). Then, for each overall data set, the response was assumed to follow a normal distribution with mean and variance given as follows:

$y_{ijkl} \sim N(\mu_{jkl}, \sigma^2)$,

where $y_{ijkl} = \mu_{jkl} + u_l + v_{kl} + w_{jkl} + e_{ijkl}$

$\mu_{jkl} = \beta_0 + \beta_1 x_{1jk} + \beta_2 x_{2jkl} + \ldots + \beta_p x_{pjkl}$,

$u_l \sim N(0, \sigma^2_u)$,

$v_{kl} \sim N(0, \sigma^2_v)$,

$w_{jkl} \sim N(0, \sigma^2_w)$, and

$e_{ijkl} \sim N(0, \sigma^2_e)$ such that $\sigma^2_u + \sigma^2_v + \sigma^2_w + \sigma^2_e = \sigma^2$,

where μ_{jkl} is the fixed effects associated with block *j* at site *k,* and species *l* where β_0 is an estimate of the model intercept and β_p are slope estimates for each linear predictor, x_{pjkl}. Then u_l is the random effect associated with the *l*th species, v_{kl} is the random effect associated with the *k*th site (within species *l*), w_{jkl} is the random effect associated with *j*th block (within species *l* and site *k*), and e_{ijkl} is the residual error associated with the *i*th response on block *j* at site *k* for species *l*.

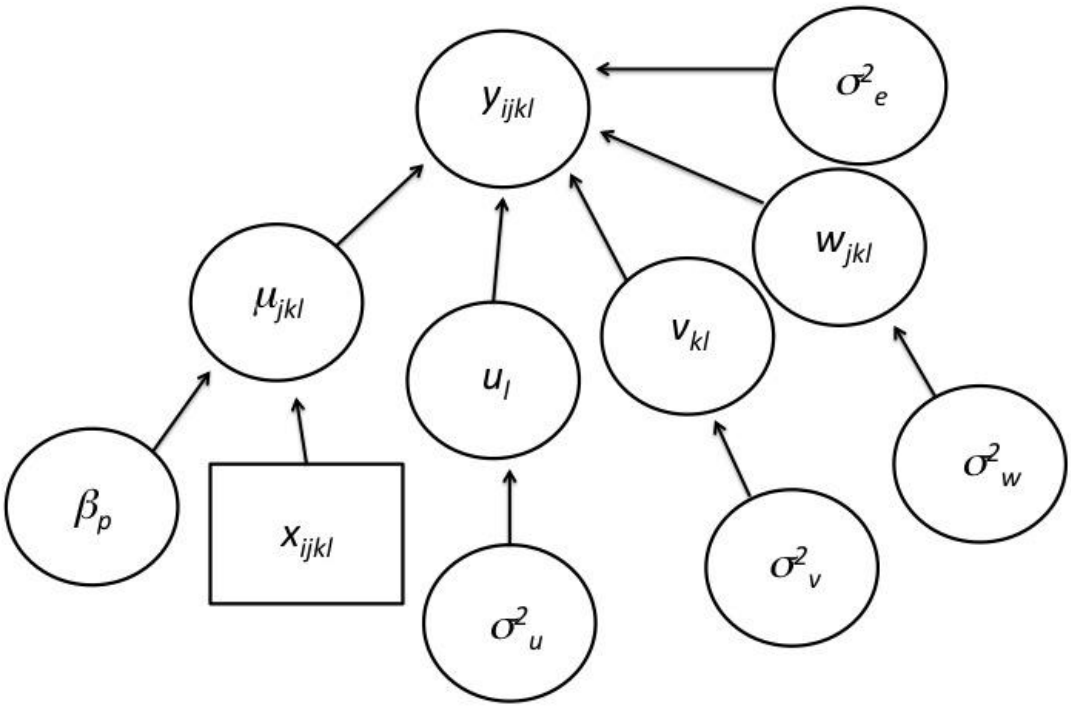

Fig. M1 Directed acyclic graph (DAG) used to represent the multilevel regression models in a hierarchical Bayesian framework for the overall model networks that were developed for both the nutrient addition experiment and the nutrient addition by herbivore exclusion experiment.

The random effect structure was constructed to reflect the design of the experiment. This structure was fixed for all models, regardless of whether each component explained a significant source of variability. Once a model was fit, residual plots were inspected for any potential relationships in the data that may not have been captured by the model (residuals were calculated as the observed value of the data minus the posterior mean prediction). Plots of the cross-validated probability integral transform (PIT, Dawid et al., 1984) for each model were also inspected. PIT values provide estimates of the probability that the

prediction is less than or equal to the corresponding observed data point, conditional on all other data. A histogram and normal quantile-quantile plot of these values were used to assess the calibration of out-of-sample predictions (Czado et al., 2009). If the residual and PIT plots were reasonable, then it was concluded that the model provided a satisfactory fit to the data.

Standardized major axis analyses description

To understand how experimental nutrient addition and fencing of vertebrate herbivores affected the scaling of leaf traits, we conducted standardized major axis (SMA) analyses for all pairs of traits considered in our study. In these analyses, we included a factor for nutrient treatments and determined whether the nutrient treatments had an effect on the elevation or slope of the trait-scaling relationships. Prior to analyses, we log-10 transformed the data for all traits. All analyses were run using R 3.3.2, using the "smart" package (Warton et al., 2011).

Structural equation models

Starting with an initial meta-model (Fig. S2) based on a priori expert knowledge and the literature, we used modification indices (Rosseel, 2012) to standardize our decisions of adding missing paths to the model. We used the "modindices" function in the lavaan package (Rosseel, 2012), which provides a list of all missing path regressions between two variables in the model, as well as the expected effect of the addition on the model data fit (Chi square value). We used the modification indices in a stepwise approach, adding ecologically sound paths one at a time, until no modification indices were higher than 2. This incremental process led to the creation of 18 different models. We then scanned path regressions and pruned all non-significant ones (based on $p<0.05$) generating a final 19^{th} model. Out of those 19 competing models, 13 had a significant model-data fit (estimated by Maximum Likelihood, Rosseel, 2012). To optimize the information-parsimony trade-off, we compared those 13 models using Akaike Information Criterion (Burnham and Anderson, 2002).

The selected best model had an AICc difference >5 with respect to the closest model 369 and an AICc weight of 0.77. To correct for the nested experimental design, we 370 included a stratified independent design with blocks nested within sites as stratified 371 variables. Using the lavaan.survey package, we extracted a robust test statistic 372 (pseudo-maximum likelihood = 23.35, 32 model degrees of freedom, and P=0.867), 373 indicating a good model-data fit. All analyses were run using R 3.3.2.

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Supplementary material

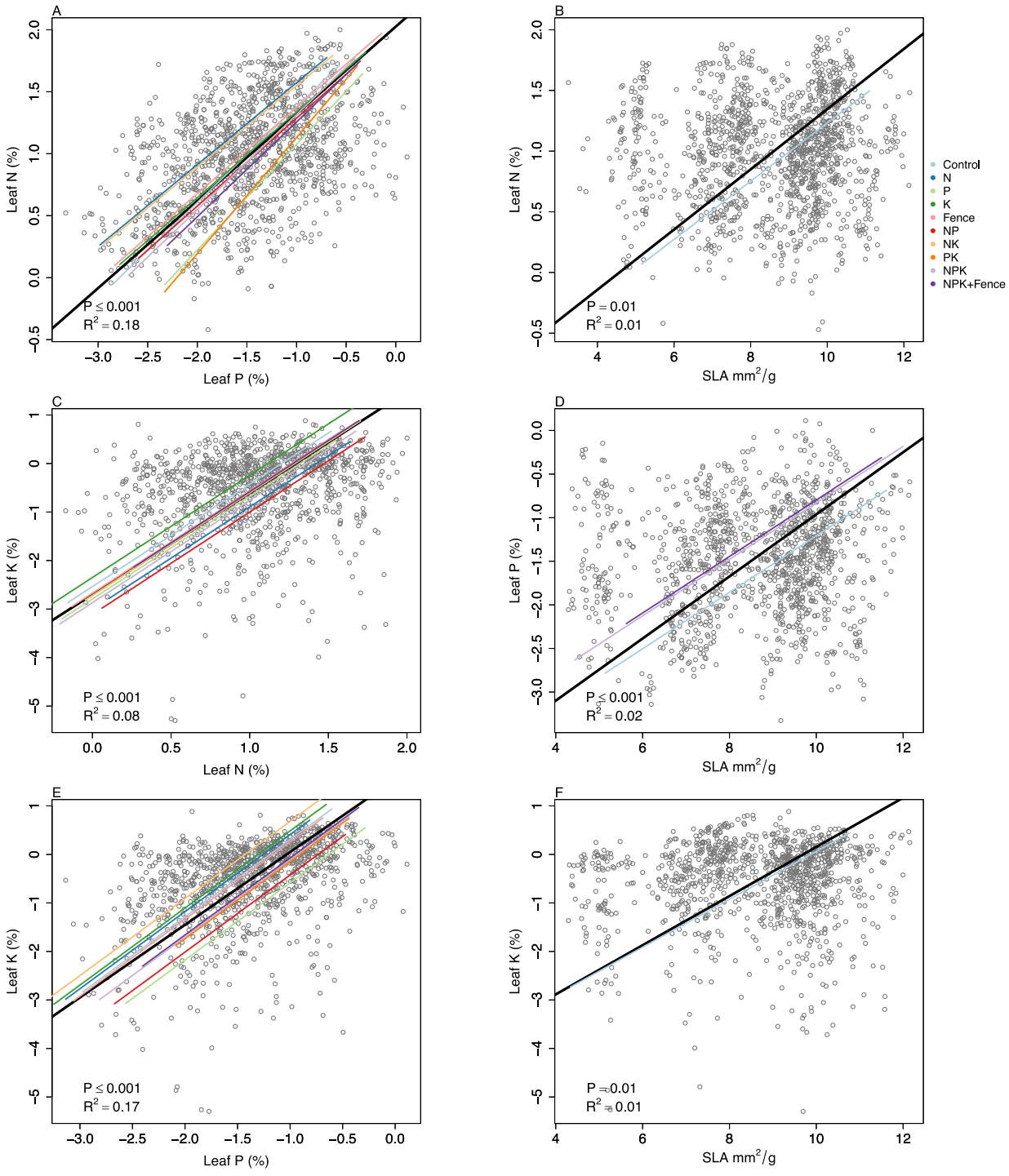

Fig. S1 Standardized major axis (SMA) relationships between leaf nitrogen content, leaf phosphorus content, leaf potassium content and specific leaf area. Solid black lines shows the overall SMA relationships, and coloured lines show SMA relationships within each of the treatments.

Structural Equation Modelling

General approach

The difficulty in building meaningful meta-models increases with the number of predictors involved because the number of potential links among variables increases exponentially. As a consequence, drawing a causal link between any two variables can have implications that are challenging to predict based on a priori knowledge (e.g., indirect effects). To reduce this level of complexity, we separated our predictors into two layers; the first one representing the experimental treatments, which are the core of the present study, and the second one representing external abiotic factors related to initial edaphic conditions, temperature and precipitation (see Methods section of the main text). We first built a meta-model that included effects from only the experimental treatments (see Fig. S2) that we tested using structural equation modelling, and then as a second step, from the knowledge gained from the first step, we built a second meta-model that integrated the effects of external abiotic factors (see Fig. S3). This sequential approach allowed us to gained sufficient insight into the system to reach a level of confidence and complexity in the final model that would otherwise have been difficult to achieve.

In both SEM analytical steps, we started with the relevant initial meta-model and used modification indices to standardize our decisions of adding missing paths to the model. We used the “modindices” function in the lavaan package, which provides a list of all missing path regressions between two variables in the model, as well as the expected effect of the addition on the model data fit (Chi-square value) (Rosseel, 2012). We used the modification indices in a stepwise approach, adding only one path at a time, until no modification indices were higher than 2. Modification indices can be constructed between any two variables in the model, and thus we only added a suggested path when it made ecological sense to do so (e.g., a path suggesting that nitrogen additionis caused by leaf nutrient content would not be considered). Once this incremental approach was finished, we scanned the path regressions and pruned all non-significant ones (based on $p<0.05$ generating a final more parsimonious candidate model. We then compared all candidate models using Akaike Information Criterion (Burnham and Anderson, 2002). This general

approach ensured that, starting from simplified meta-model, any important paths (i.e., with modification indices higher than two) between two variables would be considered and that the final selected model would represent a satisfying information-parsimony trade-off.

For all models, we corrected for the nested experimental design by including a stratified independent design with blocks nested within sites as stratified variables. Using the lavaan.survey package (Rosseel, 2012), we extracted a robust test statistic, the pseudo maximum likelihood (PML), for each model (Rosseel, 2012).

Initial step – experimental treatments only

Our initial meta-model was built based on expectations from the experimental treatments (Fig. S2). We predicted that nutrient additions would affect the leaf nutrient content and SLA directly, showing evidence of plasticity in trait expression, or through an effect on temporal species turnover, suggesting that community-level processes dominate observed effects on leaf traits (Cingolani et al., 2005; Firn et al., 2012, 2017; Funk et al., 2017,) and see Fig. S2). Temporal turnover was calculated as the Bray-Curtis dissimilarity in plot z from site y between time t0 and time x+n, which corresponded to the time of the leaf trait measurement. We started with the meta-model (Fig. S2) and followed the incremental process outlined above led, which led to the creation of 3 candidate models, from which we identified the best model with an AICc difference >13 compared with the closest model and an AICc weight of 1 (see Fig. S4). The selected model showed a very good model-data fit (PML=5.75, 15 model degrees of freedom and p= 0.98). The model showed positive effects of each nutrient on the leaf nutrient content, while only phosphorus affected plant species temporal turnover. It is noteworthy that none of the treatments had detectable impacts on SLA (Fig. S4).

Final step – integration with external abiotic predictors

Based on the insights gained during the initial step when determining the effects of the experimental treatments on the leaf traits, we built a final meta-model and integrated the effects of external abiotic factors (see Fig. S3). In this model, we assumed that if SLA was not affected by the experimental treatments then it was likely more sensitive to external

abiotic factors (Fig. S3). We also assumed that the initial soil nutrient content would affect the leaf nutrient content and that temperature and precipitation-related variables would likely influence leaf nutrients via an effect on plant species turnover (Fig. S3). This latter assumption is a simplification that allowed us to build a final meta-model that was not saturated, while integrating all predictors: given our general approach with the modification indices, we believe that it is more appropriate to start with a simplified model, assuming that all important paths (i.e., modification indices higher than 2) will be identified during the incremental process rather than starting with a saturated model where there is no space for path addition and where we have to make ad hoc decisions on which path to remove. The results from the incremental process starting with the meta-model shown in Fig. S3 are presented in Gig. 5 and in the Results section of the main text.

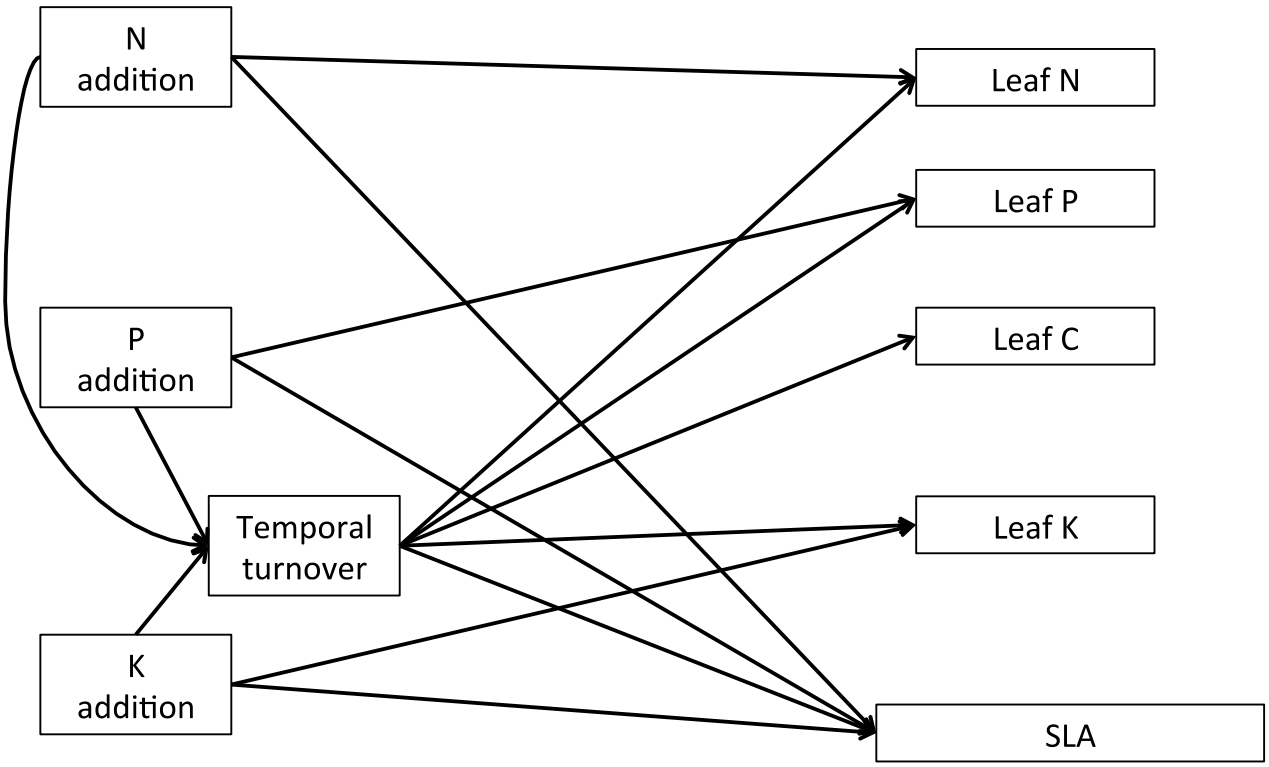

Fig. S2 Meta-model including only effects from the experimental treatments.

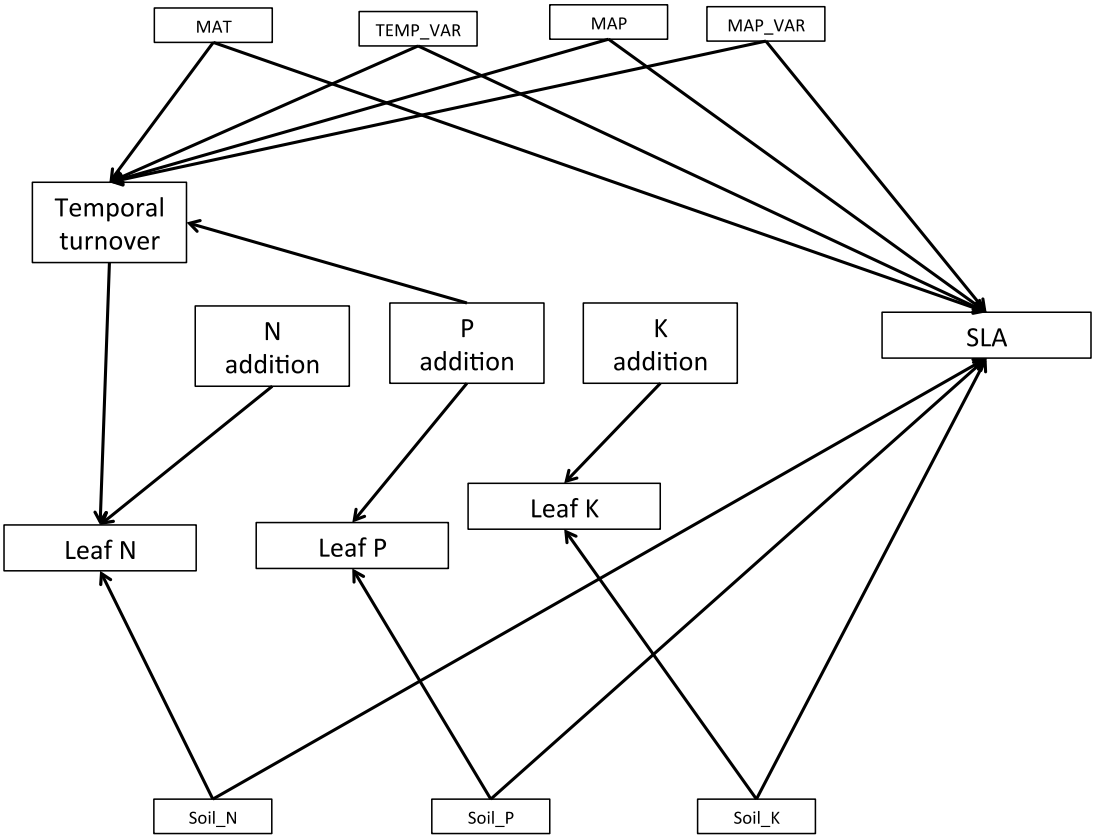

Fig. S3 Meta-model including effects from both the experimental treatments and external abiotic factors. MAT: mean annual temperature, TEMP_VAR: annual variation in temperature, MAP: mean annual precipitation, MAP_VAR: annual variation in precipitation, soil_N: initial soil nitrogen content, soil_P: initial soil phosphorus content, soil_K: initial soil potassium content.

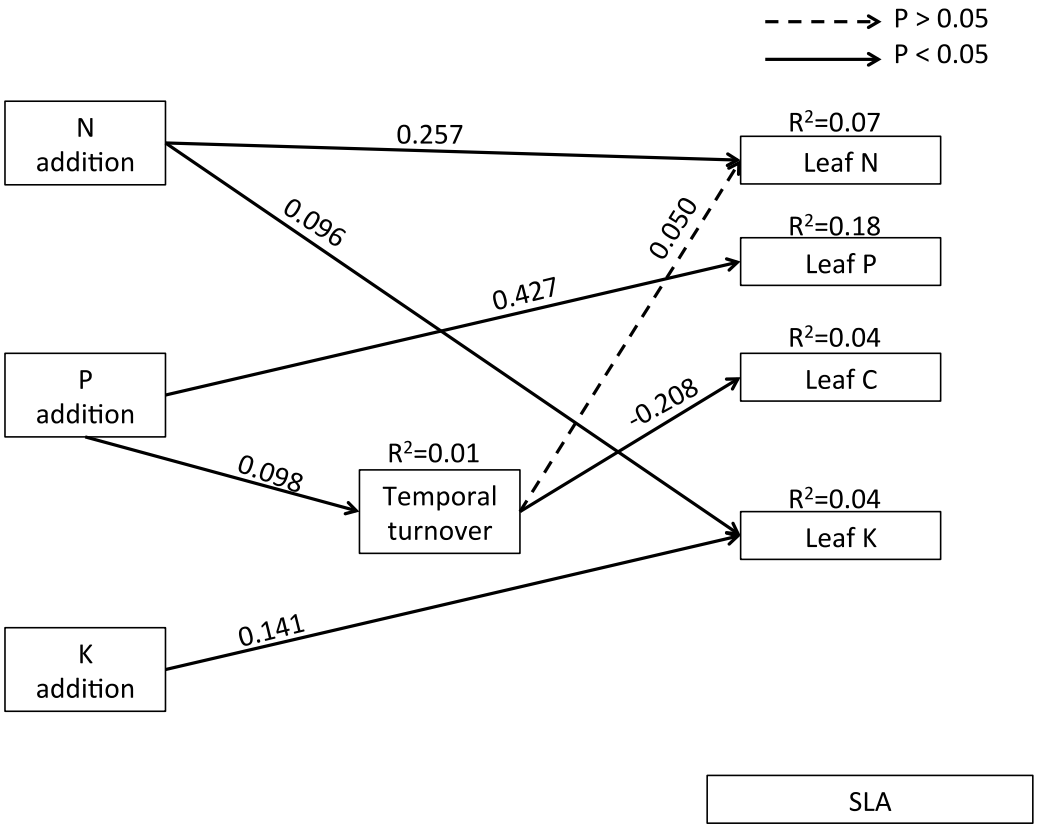

Fig. S4 Final model from the initial step including experimental treatments only. Path 646 values are standardized coefficients.

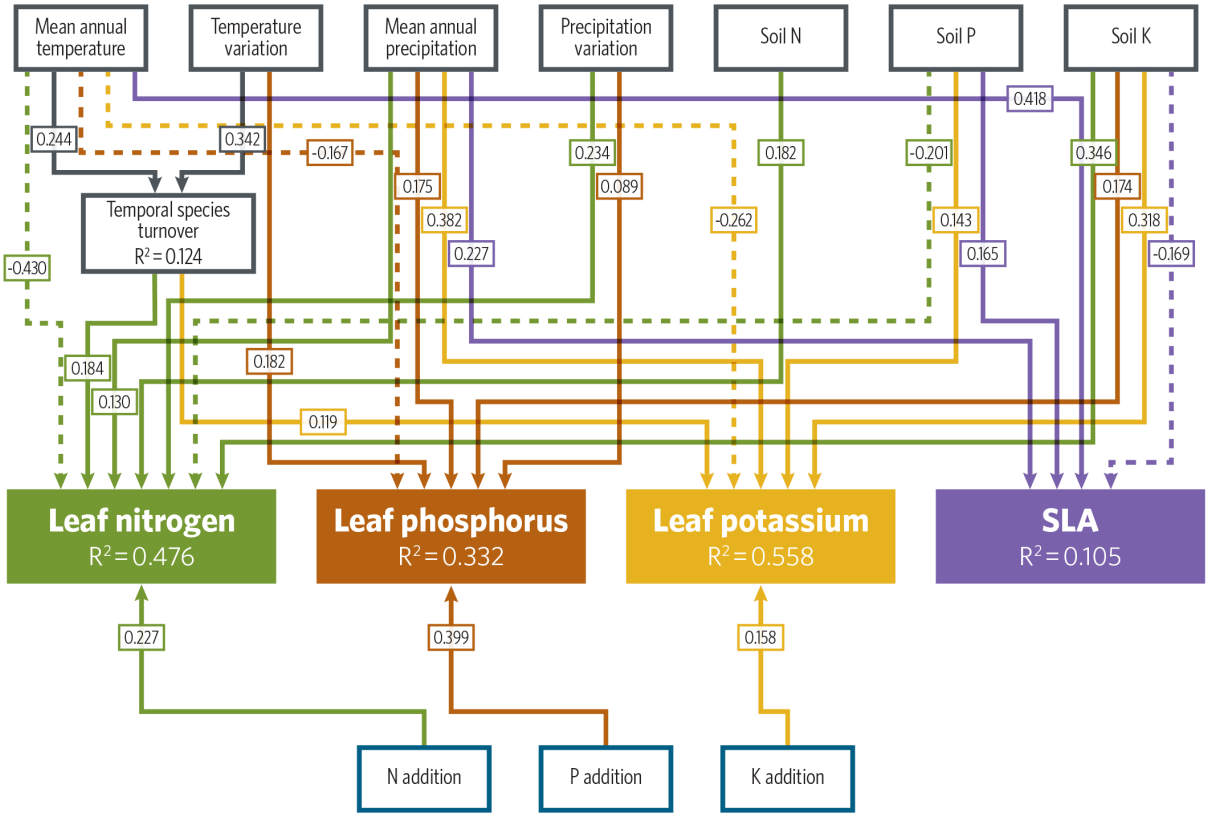

Fig. S5 Structural equation model diagram representing connections between leaf traits, experimental nutrient addition treatments, site-level average climatic and pre-treatment edaphic conditions, as well as species turnover. Values in boxes represent correlations and R^2 values. Only significant connections are shown.

Table S1 Leaf nitrogen content and leaf phosphorus content, results from standardized major axis regressions.

Treatment	Sample size (n)	R^2	*p*-value
Control	127	0.30	0.001
N	127	0.30	0.001
P	125	0.10	0.001
K	125	0.20	0.001
Fence	108	0.30	0.001
NP	126	0.20	0.001
NK	135	0.30	0.001
PK	131	0.10	0.001
NPK	124	0.20	0.001
NPK + fence	106	0.10	0.001

Table S2 Leaf nitrogen content and leaf potassium content, results from standardized major axis regressions.

Treatment	Sample size (n)	R^2	*p*-value
Control	127	0.15	0.001
N	127	0.19	0.001
P	125	0.07	0.001
K	125	0.07	0.001
Fence	108	0.19	0.001
NP	126	0.21	0.001
NK	135	0.23	0.001
PK	131	0.12	0.001
NPK	124	0.23	0.001
NPK + fence	106	0.03	0.001

Table S3 Leaf phosphorus content and leaf potassium content, results from standardized major axis regressions.

Treatment	Sample size (n)	R^2	*p*-value
Control	127	0.44	0.001
N	129	0.33	0.001
P	126	0.24	0.001
K	126	0.35	0.001
Fence	109	0.34	0.001
NP	126	0.27	0.001
NK	136	0.37	0.001
PK	132	0.29	0.001
NPK	124	0.36	0.001
NPK + fence	108	0.37	0.001

Table S4 Leaf phosphorus content and specific leaf area, results from standardized major axis regressions.

Treatment	Sample size (n)	R^2	*p*-value
Control	127	0.05	0.01
N	127	0.02	0.13
P	123	0.002	0.60
K	124	0.01	0.20
Fence	108	0.003	0.60
NP	126	0.01	0.30
NK	135	0.03	0.06
PK	131	0.002	0.60
NPK	123	0.04	0.02
NPK + fence	104	0.09	0.002

Table S5 Leaf nitrogen content and specific leaf area, results from standardized major axis regressions.

Treatment	Sample size (n)	R^2	*p*-value
Control	**139**	**0.04**	**0.02**
N	137	0.005	0.40
P	131	0.002	0.60
K	133	0.02	0.08
Fence	118	0.04	0.50
NP	136	0.00002	1.0
NK	144	0.002	0.60
PK	139	0.009	0.30
NPK	132	0.000003	1.0
NPK + fence	115	0.0002	0.70

Table S6 Leaf potassium content and specific leaf area, results from standardized major axis regressions.

Treatment	Sample size (n)	R^2	*p*-value
Control	127	0.03	0.04
N	127	0.01	0.20
P	123	0.01	0.30
K	124	0.01	0.30
Fence	108	0.01	0.30
NP	126	0.01	0.30
NK	135	0.001	0.70
PK	131	0.01	0.40
NPK	123	0.03	0.07
NPK + fence	104	0.04	0.05

Table S7 Co-variances between leaf traits from the structural equation model.

	Leaf P	Leaf K	SLA
Leaf N	0.401	0.341	0.192
Leaf P		0.462	0.224
Leaf K			0.153

Appendix 2

On estimating Gross Primary Productivity of Mediterranean grasslands under different fertilization regimes using vegetation indices and hyperspectral reflectance

Cerasoli, S., M. Campagnolo, J. Faria, C. Nogueira, and M. C. Caldeira: On estimating Gross Primary Productivity of Mediterranean grasslands under different fertilization regimes using vegetation indices and hyperspectral reflectance, Biogeosciences Discuss., https://doi.org/10.5194/bg-2018-110, in review, 2018.

Abstract

We applied an empirical modelling approach for Gross Primary Productivity (GPP) estimation from hyperspectral reflectance of Mediterranean grasslands undergoing different fertilization treatments. The objective of the study was to identify combinations of vegetation indices and bands that better represent GPP changes between the annual-peak of growth and senescence dry out in Mediterranean grasslands.

In-situ hyperspectral measurements of vegetation were collected at the same time as CO_2 gas exchange measurements were performed in control (C) and fertilized plots with added nitrogen (N), phosphorus (P) or the combination of N, P and potassium (NPK). Reflectance values were aggregated, according to their similarity (r>90%), in 26 continuous wavelength intervals (Hyp). Also, the same reflectance values were resampled reproducing the spectral bands of both Sentinel-2A Multispectral Instrument (S2) and Landsat 8 Operation Land Imager (L8) simulating the signal that would be captured in ideal conditions by either Sentinel-2A or Landsat 8.

The LEAPS procedure was applied to select the best set of the vegetation indices or spectral bands for GPP estimation using Hyp, S2 or L8. The LEAPS selected some vegetation indices putting in evidence their explanatory power as indicators of the dynamic changes occurring in community vegetation properties such as canopy water content

(NDWI) or chlorophyll and carotenoids/chlorophyll ratio (MTCI, PSRI, GNDVI) and underlining their importance for grasslands GPP estimates.

For Hyp and S2, bands showed similar explanatory power than vegetation indices to estimate GPP. A two-step LEAPS procedure allowed us also to identify spectral bands with potential for improving GPP estimates. This procedure clearly indicates the shortwave infrared region of the spectra as promising for this purpose. The comparison of S2 and L8 based models showed similar explanatory power of the two simulated satellite sensors when spectral bands were adopted.

Altogether our results show the potential of sensors on board of Sentinel 2 and Landsat 8 satellites for monitoring grasslands phenology and improving GPP estimates in support of a sustainable agriculture management.

Introduction

Mediterranean grasslands are high biodiverse ecosystems, covering around 22% of the European Union land area, and providing important ecosystem services such as forage production (Bugalho and Abreu, 2008; Díaz-Villa et al., 2003). These ecosystems are subjected to large pressures under global change (Sala, 2000), namely by the increasing availability of nutrients (e.g., phosphorus (P) and nitrogen (N)) due to human use of fertilizers, and N deposition (Ceulemans et al., 2014; Galloway et al., 2004; Peñuelas et al., 2013) and by a decrease and shift in seasonal patterns of precipitation (Costa et al., 2012; Kovats et al., 2014). The contemporary changes in water and nutrients supply can affect species composition, biomass, phenology along the life cycle of annual grasslands (Harpole et al., 2007), compromising their productivity. In particular, the onset and extension of the senescence period, largely dependent on soil water availability, can be affected in Mediterranean grasslands with great impacts in their functioning (Aires et al., 2008a, 2008b; Jongen et al., 2013; Xu and Baldocchi, 2004).

Using remote sensing based information to evaluate GPP brings important advantages both from a scientific and management point of view. Spectral retrievals collected from optical sensors on board of remote platforms may provide information on many biophysical properties of vegetation and can be usefully employed for monitoring and modelling ecosystems GPP in a cost and time-effective way (Schimel et al., 2015). Also, for land

managers, the capability of making timely grassland management decisions may improve the use and sustainability of these ecosystems.

GPP estimation models integrating remote sensed observations increased considerably in the last decades (Beer et al., 2010; Grimm et al., 2008). Such models are generally based on the Light Use-Efficiency (LUE) concept (Monteith, 1972, 1977), which defines GPP as a function of the fraction of radiation absorbed by vegetation (*f*PAR), which in turn depends on green leaf area and the efficiency by which light energy is used to fix carbon during photosynthesis (i.e. LUE) (Cheng et al., 2014; Yuan et al., 2014).

Based on this approach large efforts have been put to derive vegetation indices able to represent the green leaf area and LUE. The NDVI is widely used for its known linear relationship with fPAR (Fensholt et al., 2004; Joel et al., 1997; Myneni and Williams, 1994). However, some exceptions are reported in the literature. For example in highly productive environments, such as grasslands, NDVI becomes easily saturated, not responding to increased leaf area and LUE, and the regression observed is no more linear (Vescovo et al., 2012; Viña and Gitelson, 2005).

In annual grasslands, such as the Mediterranean, control on ecosystem carbon balance is generally considered related mainly to the amount of green leaf area, while little LUE changes are expected (Gamon, 2015). Nonetheless, several studies reported a hysteresis in LUE in grasslands when the duration of the study encompasses the whole life cycle (Nestola et al., 2016; Perez-Priego et al., 2015).

The Photochemical Reflectance Index (PRI) is frequently adopted as a proxy of LUE (Gamon et al., 1997; Peñuelas et al., 1995). PRI in the short term mirrors the dynamic of the xanthophylls cycle (Peñuelas et al., 1995) which is related to thylakoid energization and hence to light harvesting by photosynthesis. In the long term was found to be correlated with the ratio of carotenoids to chlorophyll (Filella et al., 2004; Porcar-Castell et al., 2012) and hence to plant senescence, since chlorophyll degradation and N export is a distinctive process of leaf ageing (Thomas, 2013). However, also PRI shows some drawbacks, since it is largely affected by species identity, leaf age or environmental conditions (Peñuelas et al., 1995) and by sensors geometry and atmospheric factors (Moreno et al., 2012). Hence the performance of models integrating PRI is frequently below the expected (Perez-Priego, 2015).

As a result, other vegetation indices have been tested as alternatives to NDVI and PRI for GPP estimation. Rossini et al. (Rossini et al., 2012), in an subalpine grassland obtained the best model to estimate GPP adopting together the MERIS Terrestrial Chlorophyll Index (MTCI) (Dash and Curran, 2004), a proxy of chlorophyll, and PRI. In another study, in a subalpine grassland, Sakowska (Sakowska et al., 2014), found that the red-edge NDVI, a modified NDVI, where the infrared band is substituted with a red-edge band (Gitelson and Merzlyak, 1994) improved GPP estimates. In Mediterranean grasslands with different N and P fertilization level, PRI together with solar induced fluorescence improved GPP estimates (Perez-Priego et al., 2015). In a semi-arid grassland Vicca et al. (Vicca et al., 2016) observed that several vegetation indices including NDVI and the Normalized Different water Index (NDWI) (Gao, 1996), a proxy of vegetation water content, were able to capture the drought effect on GPP.

Altogether these results clearly indicate the need for further studies aiming to identify the vegetation indices and the regions of the spectra of potential interest for GPP estimates of grasslands under different environmental constraints, such as nutrients availability.

The adoption of a specific model and vegetation index depends also frequently on the availability of remote sensed products at a suitable spatial and temporal scale. In the case of local scale monitoring of managed grasslands, sensors with high spatial resolution will produce better results than sensors with coarse spatial resolution. In this study we opted for using data from Sentinel-2A MSI (Multi-Spectral Instrument), (hereafter named S2) and Landsat8 OLI (Operational Land Imager) (hereafter named L8), for their spatial resolution, (10-20m for S2 and 30m for L8) more suitable for representing grasslands spatial heterogeneity and hence better adapted to implement management options from a precision agriculture perspective. The L8 provides reflectance in 7 bands ranging from the visible to the short wave infrared region (SWIR) (Loveland and Irons, 2016), but its main drawback is the long revisiting time of 16 days. The recently launched S2 covers the regions of the visible and near-infrared and the SWIR in 13 bands with at least five days revisiting time when both S-2A and S-2B platforms will become operational (Drusch et al., 2012).

Field collection of vegetation reflectance by hyperspectral sensors is less cost-effective and more time consuming than satellite remote sensed data but presents the advantage of providing reflectance in numerous, high resolution, wavelengths (Porcar-Castell et al.,

2015). Therefore, it can be usefully employed for identifying which wavelengths best mirror biophysical properties and physiological status of vegetation (Balzarolo et al., 2015; Matthes et al., 2015) and put in evidence regions of the spectra of potential interest for GPP modelling actually not exploited by remote sensors. The high detail of spectral resolution (1 nm nominal) is a further advantage of hyperspectral measurements. In particular, it allows comparing the performance of similar vegetation indices available from different satellite platforms resampling hyperspectral information to match spectral bands of different remote sensors.

The aim of this study was to identify combinations of vegetation indices and bands that better represent GPP changes in the period comprised between the annual-peak of growth and senescence dry out in Mediterranean grasslands subjected to different fertilization treatments.

To achieve this goal, in situ hyperspectral measurements of vegetation reflectance were employed to estimate GPP in Mediterranean grasslands before and after the annual peak of growth was achieved. A set of vegetation indices proposed in the literature were calculated and the performance of models to estimate GPP based on linear combinations of vegetation indices and bands were compared.

Whenever possible, vegetation indices were also calculated simulating S2 and L8 bands and the performance of GPP estimates based on remote platforms and in situ hyperspectral measurements compared.

The specific objectives of the study were: (i) Identify a set of vegetation indices useful to optimize a GPP model for Mediterranean grasslands; (ii) Compare the performance of GPP models employing vegetation indices only and in combination with spectral bands; (iii) Finally, compare GPP models using spectral information obtained from hyperspectral sensors with similar models obtained from S2 and L8 platforms.

Material and Methods

The study site

Our study was conducted in a semi-natural Mediterranean grassland at Companhia das Lezírias, an estate of approximately 15 000 ha, located north-east of Lisbon, Portugal

(38°49´45.13´´N, 8°47´28.61´´W). The grassland plant community is composed mainly of annual C3 species. The climate is Mediterranean, with mild, wet winters and hot, dry summers. Long-term (1961–1990) mean annual rainfall is 709 mm. Mean annual temperature is 15.9 °C (INMG, 1991). Site topography is flat and the soil is a well-drained deep Haplic Arenosol (WRB, 2006).

Experimental design

The grassland studied is part of the Nutrient Network experiment (http://www.nutnet.umn.edu; Borer et al., 2017; Seabloom et al., 2013). Plots (5m x 5m) were established in 2012, in a randomized block design. Factorial combinations of nitrogen (N), phosphorus (P), and potassium plus micronutrients (K), a total of eight treatments per block, including the control (C) with no added nutrients, were considered. All nutrients were added at a rate of 10 g. N. m^{-2} yr^{-1}. N was added as slow-release urea (60-90 days), P was added as triple-super phosphate and K as potassium sulphate. Micronutrients (6% Ca, 3% Mg, 12% S, 0.1% B, 1% Cu, 17% Fe, 2.5% Mn, 0.05% Mo, and 1% Zn) were added with K only once, at the start of the study to avoid possible micronutrient toxicity. In this study, only four fertilization treatments were considered: C, N, P and NPK. Each one of these treatments was repeated twice per block, a total of 24 plots were considered (2 replicates X 4 treatments X 3 blocks).

Environmental measurements

Temperature, PAR and relative humidity were measured in situ using a VP-3 humidity temperature and vapour pressure sensor and QSO-S PAR Photon Flux sensor (Decagon Devices, Pullman, USA) logged every 30 min (EM50 data logger, Decagon Devices, Pullman, USA). Precipitation was recorded using a tipping bucket rain gauge (RG2, Delta-T Devices, Cambridge, UK). Soil water content (SWC) was continuously measured, at a depth of 10 cm, using EC-5 soil moisture sensors (Decagon Devices, Pullman, USA). The rain gauge and soil sensors were connected to a CR1000 and AM16/32B multiplexer data logger (Campbell Scientific, Logan, USA).

Field Measurements

NEE and R from a closed system IRGA

Grassland net ecosystem exchange (NEE) was measured with a closed chamber (40 cm X 40 cm X 54 cm) of polymethylmethacrylate (3 mm thick) inserted into a permanent frame buried 5 cm into the soil. Radiation transmittance was higher than 95%. The same chamber was covered with a reflective cloth for dark respiration (R) measurements. Air temperature inside the chamber was continuously monitored and PAR was measured at beginning and end of measurements with a ceptometer (AccuPAR-LP80, Decagon Devices, Inc. Pullman, WA, USA). Fans in the chamber ensured air circulation. The chamber was connected to an infrared gas analyser (LI-840, Li-Cor, Lincoln, NE, USA) measuring CO_2 and water vapour. Each measurement was no longer than 3 min. Fluxes were calculated based on the rate of change of CO_2 inside the chamber, after an initial period of at least 10 seconds. Flux calculations and corrections for CO_2 water vapour dilution followed Perez-Priego (2015). GPP was obtained by detracting R from NEE at each measurement. All plots were measured between 11:00 and 13:00 on clear sky sunny days. Measurements were performed during the 2016 growing season. Two field campaigns were carried out during vegetation growth, day 1 (31st March to 1st April) and day 2 (24th to 25th April) and two during the senescence phase, day 3 (19th – 20th May) and day 4 (1st-3rd June).

Leaf area and biomass

The Plant Area Index (PAI) was indirectly measured with a linear PAR ceptometer (AccuPAR LP-80 Decagon Devices Inc., Pullman, WA, USA). The ceptometer measures the fraction of PAR intercepted by the canopy (*f*PAR) according to equation (1):

$$fPAR = \frac{1-PAR_t}{PAR_i}$$

(1)

The *f*PAR was considered approximately equal to absorbed radiation, as the amount of reflected radiation in the PAR range is usually low (Gower et al., 1999). For each plot, 6-8 measurements above (PAR_i) and below (PAR_t) the canopy were taken and averaged.

The PAI is calculated by inversion of the Beer-Lambert law (equation 2):

$$fPAR = 1 - e^{-K*PAI} \quad (2)$$

where K is the light extinction coefficient, which depends on the leaf angle distribution of the canopy, in this study considered spherical distributed, and on the zenith angle of the probe, calculated by the ceptometer with basis on the geographic coordinates of the local and date and time of measurements. To avoid low solar zenith angles all measurements were performed around solar noon. As the growing season progressed some species started to senesce. In order to estimate the fraction of PAR absorbed only by photosynthesizing components of the canopy ("green" PAI, PAIgr), PAI was multiplied by a normalized (by scaling between 0 and 1) greenness index (GI, calculated as a ratio between the digital number values of green and the sum of red, green, and blue digital number values) derived from the analysis of digital pictures of the plots taken at each measurements day around solar noon (Cyber-shot DSC-W530, SONY), using the Phenopix R package (Filippa et al., 2016).

A strip of vegetation (0.1 mx1 m) within each plot was also collected close to the peak growth and biomass divided into functional types (legumes, forbs, graminoids) and dried in an oven.

Hyperspectral measurements of vegetation reflectance

At each field campaign, hyperspectral observations of all plots were also acquired with a FieldSpec3 spectroradiometer (ASD Inc., Boulder, USA), which provides reflectance of vegetation in the range of 350-2300 nm. The spectral resolution (Full-Width-Half-Maximum) is 3 nm at 700 nm and 10 nm at 1400 nm and 2100 nm. The sampling interval is 1.4 nm for the spectral region of 350-1000 nm (visible and near infrared) and 2 nm for the spectral region of 1000-2500 nm (short-wave infrared). Spectra at 1nm intervals are obtained from a cubic spline interpolation function. Five spectra were collected for each plot, each representing the average of 25 spectra, employing a bare fibre optic cable (with an instantaneous field of view of 25^{o}) inserted into a pistol grip at approximately 90 cm above the canopy. A white reference of known reflectance (Spectralon panel, Labsphere, Inc., North Sutton, USA) was used to normalize for variations in atmospheric conditions

and to convert the measurements into absolute reflectance (Ref.). All measurements were conducted immediately after grassland gas exchange measurements, within two hours around solar noon, to minimize the effects of shadowing and solar zenith changes.

Data analysis

All statistical analyses were performed using open-source R (R Core Team, 2016). We used the lme4 package (Bates et al., 2014) to perform linear mixed effect analyses of the effect of the fertilization and control treatments on NEE, R, GPP and PAIgr. Treatment and date were the fixed effects and the block was the random effect. Conditions of homoscedasticity and normality were always verified by visual inspection for residuals. P-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. A Tukey test was used for post-hoc comparison using the multcomp package (Hothorn et al., 2008).

The full spectra of vegetation reflectance retrieved from the Fieldspec was used to model GPP, after excluding noisy values in the range 1350-1400 nm and 1800-1950 nm. Our P=1748 original explanatory variables are $x_{350},...,x_{2299}$ where x_λ represents the reflectance in the narrow band [λ, λ +1] (nm) and our response variable is the GPP (□mol m^{-2} s^{-1}). A total number of 96 observations were available (4 treatments X 2 replicates X 3 blocks X 4 dates). Since we have 1748 explanatory variables and just 96 observations, hence a high level of redundancy in our data, the dimensionality was reduced by grouping variables that belong to intervals of wavelengths where all variables are highly correlated. A hierarchical cluster analysis was performed to reduce the number of predictors from P=1748 to P=25 groups of contiguous variables named Bands. The distance between two variables is the correlation coefficient and the distances within a band is given by the complete link criterion to guarantee that $r(x_{\lambda a}, x_{\lambda b})>0.90$ for any pair of variables ($x_{\lambda a}$, $x_{\lambda b}$) within each group.

Formally, wherever all pairs of variables $x_{\lambda a}$ and $x_{\lambda b}$ -- such that $\lambda_1 \leq \lambda_a < \lambda_b \leq \lambda_2$ – are highly correlated, i.e. $r(x_{\lambda a}, x_{\lambda b})>0.9$, then the original variables $x_{\lambda 1},\ldots,x_{\lambda 2}$ in the interval [λ_1, λ_2] are replaced by a new variable $x_{[\lambda 1, \lambda 2]}$, which is the arithmetic mean of $x_{\lambda 1},\ldots,x_{\lambda 2}$.

Reflectance values were also resampled to simulate bands of Sentinel-2A MSI (S2) and Landsat8 OLI (L8). For each sensor and band [λ_1, λ_2], we calculated the response as a

weighted mean of $x_{\lambda 1},\ldots,x_{\lambda 2}$, where the weights are the coefficients of the spectral response function (Barsi et al., 2014; ESA, 2018). The list of S2 and L8 bands used in this study is shown in table 1.

Table 1 Spectral bands range and spatial resolution of Sentinel-2A MSI and Landsat 8 OLI sensors simulated in this study.

Band	Sentinel-2A MSI			Landsat 8 OLI		
	Spectral region	**Wavelength range (nm)**	**Resolution (m)**	**Spectral region**	**Wavelength range (nm)**	**Resolution (m)**
B1				Blue	435-451	30
B2	Blue	458-523	10	Blue	452-512	30
B3	Green peak	543-578	10	Green	533-590	30
B4	red	650-680	10	Red	636-673	30
B5	Red-edge	698-713	20	NIR	851-879	30
B6	Red-edge	733-748	20	SWIR1	1566-1651	30
B7	Red-edge	773-793	20	SWIR2	2107-2294	30
B8	NIR	785-899	10			
B8A	NIR narrow	855-875	20			
B11	SWIR	1565-1655	20			
B12	SWIR	2100-2280	20			

Vegetation indices (VIs) (Table 2) were calculated from hyperspectral (Hyp), or simulated S2 and L8 sensors (Table 1). The VIs were selected from the literature with basis based on their relation to biophysical properties of vegetation affecting GPP. The NDVI and the NDVIre, are considered a proxy of fPAR; the Green Normal Difference Vegetation Index (GNDVI), the MERISTerrestrial Chlorophyll Index (MTCI) and the chlorophyll index (CI) are representative of chlorophyll-a and N content, while the Photochemical Reflectance Index (PRI) and the Plant Senescence Reflectance Index (PSRI) are expected to mirror changes in the ratio of carotenoids to chlorophyll. Finally, the Normalized Difference Water Index (WBI) and the Normalized Difference Water Index (NDWI) are considered proxy of tissue water content.

Table 2 Selection of vegetation indices adopted in this study with their formulation using hyperspectral (Hyp) grouped bands, S2 or L8 simulated sensors, biophysical properties represented according to the literature and original bibliographic reference.

Vegetation Index		Hyp	S2	L8	Biophysical property	Reference
NDVI	Normalized Difference Vegetation Index	$\frac{R_{800}-R_{670}}{R_{800}+R_{670}}$	$\frac{B8A-B4}{B8A+B4}$	$\frac{B5-B4}{B5+B4}$	Green biomass and area	(Rouse et al., 1974)
GNDVI	Green Normalized Diff. Veg. Ind.	$\frac{R_{750}-R_{550}}{R_{750}+R_{550}}$	$\frac{B7-B3}{B7+B3}$	$\frac{B5-B3}{B5+B3}$	Green biomass and area	(Gitelson and Merzlyak, 1998)
NDVIre	Red-edge Normalized Diff. Veg. Ind.	$\frac{R_{750}-R_{720}}{R_{720}+R_{750}}$			Green biomass	(Gitelson and Merzlyak, 1994)
CI	Chlorophyll index	$\frac{R_{750}-R_{705}}{R_{750}+R_{705}}$			Chlorophyll	(Gitelson and Merzlyak, 1994)
MTCI	MERIS Terrestrial chlorophyll Index	$\frac{R_{754}-R_{709}}{R_{709}+R_{681}}$	$\frac{B6-B5}{B5+B4}$		Chlorophyll, nitrogen	(Dash and Curran, 2004)
PRI	Photochemical Reflectance Index	$\frac{R_{570}-R_{531}}{R_{570}+R_{531}}$			Radiation Use-Efficiency, Carotenoid/chlorophyll	(Gamon et al., 1992)
PSRI	Plant Senescence Reflectance Index	$\frac{R_{680}-R_{500}}{R_{750}}$	$\frac{B4-B3}{B6}$		Carotenoid/chlorophyll	(Merzlyak et al., 1999)
NDWI	Normalized Difference Water Index	$\frac{R_{860}-R_{1240}}{R_{860}+R_{1240}}$			Tissue water content	(Gao, 1996)
WBI	Water Band Index	$\frac{R_{970}-R_{900}}{R_{970}+R_{900}}$			Tissue water content	(Penuelas et al., 1993)

A multiple linear regression (MLR) was adopted to model the relation between our explanatory variables (bands and VIs) and the response variable (GPP). Since the number of observations is only roughly twice as large as the number of new explanatory variables we performed a variable selection and excluded variables that do not contribute significantly to the goodness-of-fit of our model. Although the dimensionality of the problem is very large, it can be solved efficiently by the LEAPS algorithm (Furnival and Wilson, 1974) available through the R package leaps (Lumley, 2009).

A nested approach was adopted to formally test which model better explained GPP. A preliminary test showed that better results were obtained with exponential regressions and therefore lnGPP was adopted as the response variable in all analyses.

The general model was ln $GPP \sim \sum_{j,1}^{n} vj$, where v are vegetation indices (VIs) or optical bands (B) from Hyp grouping procedure or from simulated S2 or L8 data. The subset of $\square_j$ was selected by maximizing the adjusted R^2 among all possible combination of predictors.

The LEAPS procedure returns and optimal model named L. However, L may include variables which contribute only marginally for the overall adjusted R^2. To further reduce the dimensionality of the predictors, we test sub-models of L (obtained by backwards stepwise selection of predictors) against the LEAPS optimal model L. When sub-models of L were found not to be significantly worse than L, at a significance level alpha=0.05, then we considered the most parsimonious of those sub-models as the optimal solution. A F-test was used to perform those comparisons. The analysis was repeated separately for all vegetation indices (VIs) and bands (B) from Hyp, S2 or L8 data, obtaining an optimal model for each sensor.

Besides determining the adjusted R^2 for the optimal model from the full sample, we applied a bootstrap procedure (N=10000 iterations) to estimate the distribution of the adjusted R^2 in the whole population (Ohtani, 2000). This allowed us to estimate quantiles (25%-75%) for adjusted R^2 and also compare the adjusted R^2 distributions among models. In particular, it permits to estimate the probability that some model A has a higher adjusted R^2 than an alternative model B.

Two-sstep models were also used to investigate if optical bands had the potential to improve models based only on vegetation indices (VIs). Toward that end, bands (B) were added to the optimal models obtained by the procedure above described denoted by Hyp-VIs, S2-VIs and L8-VIs (step 1). Using step 1 as the base model, we applied LEAPS to determine the subset of bands that maximized the overall adjusted R^2. As before, we applied a F-test (alpha=0.05) to possibly reduce the number of bands in the optimal model. As a result, we defined the optimal two-sstep models: Hyp-VIs+B, S2-VIs+B and L8-VIs+B. Finally, for Hyp, S2 and L8, we performed a F-test to compare the one step optimal model with the correspondent two-ssteps optimal model. A low p-value for this F-test indicates that the two-step model is significantly better and means that bands, in addition to vegetation indices, contribute for an improved modelling of GPP.

Results

Conditions during the experimental period

During the period of measurements, from March 31 to June 3, the average daily PAR and temperature increased progressively, ranging from 630 µmolm^{-2}s^{-1} to 1000 µmolm^{-2}s^{-1} and from 9.6 ˚C to 17˚C, respectively (Fig.1a). Soil water content (SWC) (Fig. 1b) showed fluctuations according to rainfall events, ranging from 0.05 to 0.2 m^{3}m^{-3}. During the experimental period, rainfall was concentrated in the first half of April and at the beginning of May. Along the experimental period rainfall recorded was 195mm, corresponding to the 33% of the whole year.

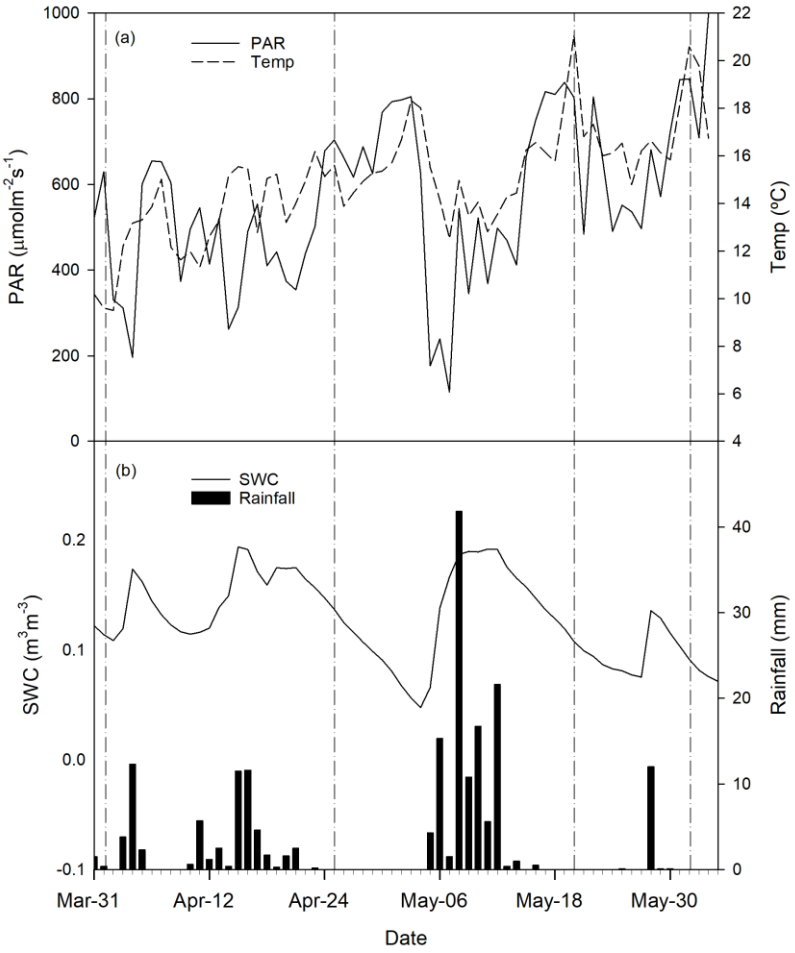

Fig 1 Daily average PAR, temperature (a), soil water content for different treatments and total rainfall (b) recorded on the site during the experimental period. Dates of field measurements are indicated by vertical dash-dotted lines.

The effect of fertilization on leaf area and species composition

From the beginning to the end of the study period, PAI increased on average 4 fold from 1 to 4 (Fig. 2a). In all treatments, the increase in PAI was completed by May 20 and no further increase was observed in the last measurement (June 3). On the contrary, at the beginning of the experiment PAIgr (Fig.2b) showed an increasing tendency similar to PAI but from May 20 onwards, the trend changed and a decreasing trend was then observed corresponding to the onset of grassland senescence.

The fertilization treatments influenced both the PAI ($P<0.000$) and the PAIgr (Fig. 2b, PAIgr) ($P<0.000$) being both significantly higher for treatments NPK and P than for treatment C ($P<0.001$). No differences were observed between C and N treatments ($P>0.05$). In both PAI and PAIgr the treatment P showed similar values to NPK, with the exception of the first measurements day (April 1). The grassland communities fertilized with NPK had a higher and earlier leaf area growth when compared to the other treatments.

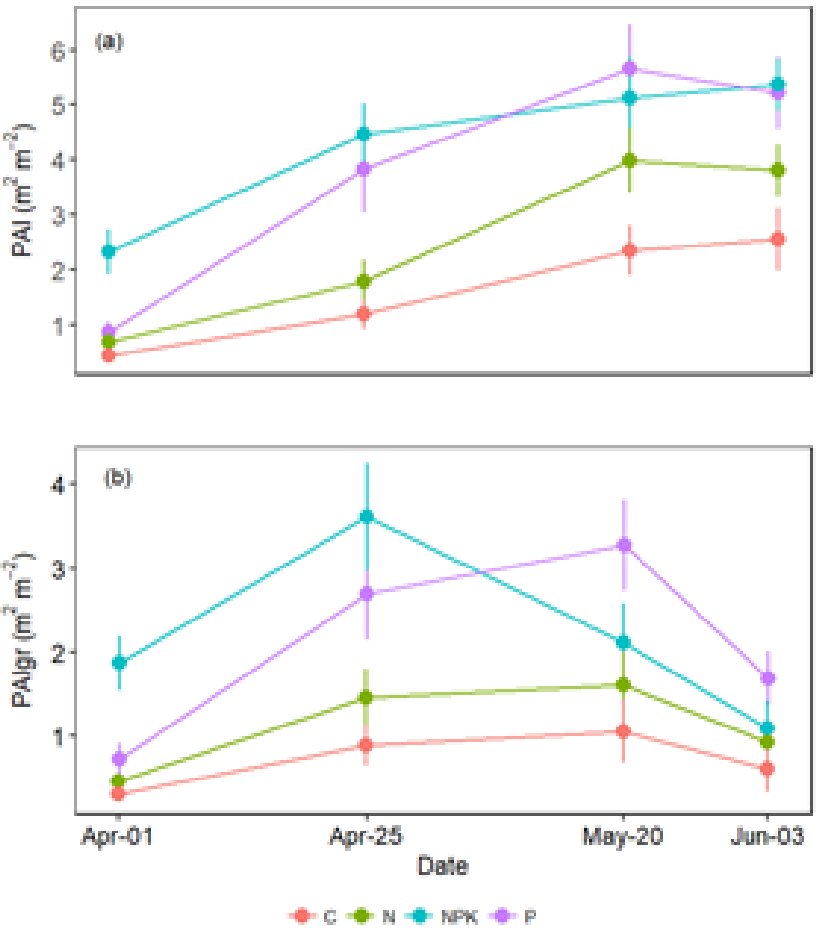

Fig. 2 Average green Plant Area index (PAI) and the green fraction of PAI (PAIgr) observed in grasslands subjected to different fertilization treatments (C, N, NPK or P). Each point is the average of 6 replicates. Vertical bars represent error bars.

The fertilization treatments also influenced the functional composition of grasslands (Table 3). In the NPK treatment the percentage of graminoids was higher than in any of the other treatments. P treatment showed a higher percentage of legumes and in the C and N treatments forbs were the dominant functional group.

Table 3 Percentage of each plant functional type (Forbs, Graminoids and Legumes) in above ground biomass of grasslands under different fertilization treatments (C, N, NPK and P). Values represent means of 6 replicates; standard errors are shown in parenthesis.

Treatment	Forbs	Grams	Legumes
C	56.85 (5.10)	21.22 (3.62)	21.93 (4.20)
N	65.00 (1.89)	25.04 (2.53)	9.95 (1.45)
NPK	34.07 (3.43)	52.55 (3.29)	13.37 (1.03)
P	25.60 (3.06)	31.43 (4.47)	42.96 (3.82)

The effect of fertilization on GPP

The ability of grasslands to sequester atmospheric carbon dioxide was not affected by fertilization treatments. The NEE (Fig. 3a) and the GPP (Fig. 3c) did not reveal any statistical significant difference among treatments ($P>0.05$). On the contrary, the rate of respiration (Fig. 3b, R) was affected by the fertilization treatment ($P<0.05$), being on average higher for treatments NPK and P than C. CO_2 gas exchanges were influenced by the grassland life cycle and marked trends were observed along the measurement period. NEE showed an average drop of 74%, shifting from -14.47 $\mu molm^{-2}s^{-1}$ to -3.67 $\mu molm^{-2}s^{-1}$, from April 01 (day 1) to June 03 (day 4) ($P<0.000$). This decrease in NEE rate was particularly evident from the second to the third measurement day, after the annual peak of grassland growth was achieved (Fig. 3a). R also showed differences along the experimental period ($P<0.000$) but the trend observed was different. R increased from the first to the second measurement day, from 8.22 $\mu molm^{-2}s^{-1}$ to 13.65 $\mu molm^{-2}s^{-1}$ and then decreased toward the end of the experiment (Fig. 3b). GPP also changed significantly along the studied period ($P<0.000$), decreasing from 25.72 $\mu molm^{-2}s^{-1}$ on April 25 (day 2) to 12.12 $\mu molm^{-2}s^{-1}$on June 3(day 4).

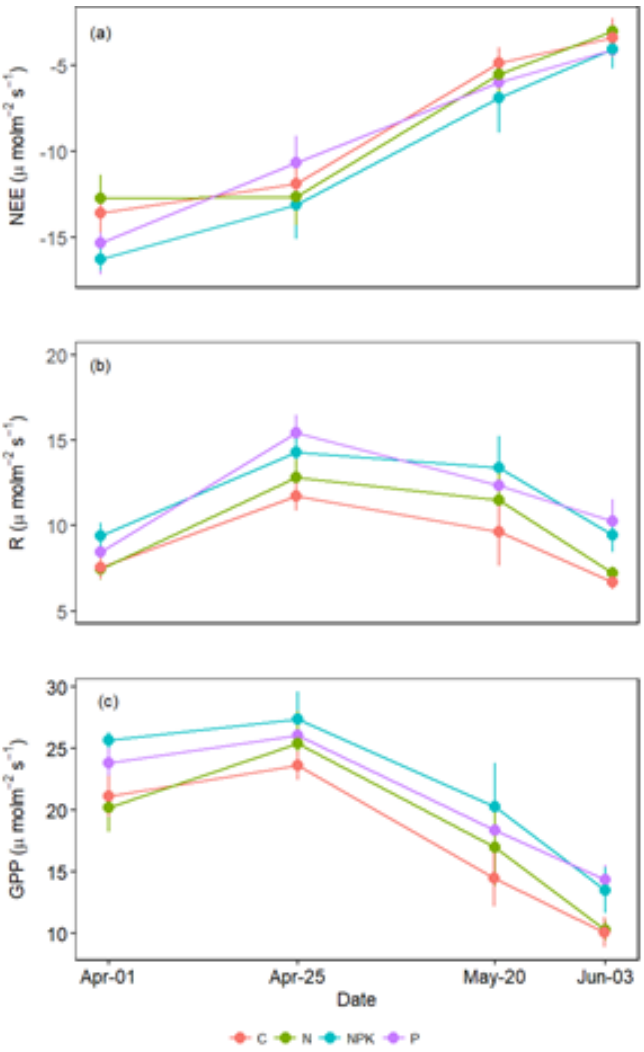

Fig. 3 Average Net Ecosystem Exchange (NEE)(a), dark Respiration (R) (b) and Gross Primary Productivity (GPP) (c) measured in grasslands under different fertilization regimes (C, N, NPK and P). Each point is the average of 6 replicates. Vertical bars represent standard errors.

Vegetation reflectance

The reflectance of vegetation (Ref) varied on average between 0 and 0.4 (Fig. 4a).The Cluster analysis created 25 bands (Fig. 4b) based on Ref similarity of contiguous wavelengths (r>90%). Bands were narrower in the visible region (350 nm to 750 nm) than in the NIR (750 nm to 1350 nm) and in the SWIR (1350 nm to 2300 nm) region. In particular, in the red-edge region, between 698 and 732nm 6 different bands were identified, corresponding to a steep increase in reflectance observed in this region of the spectra.

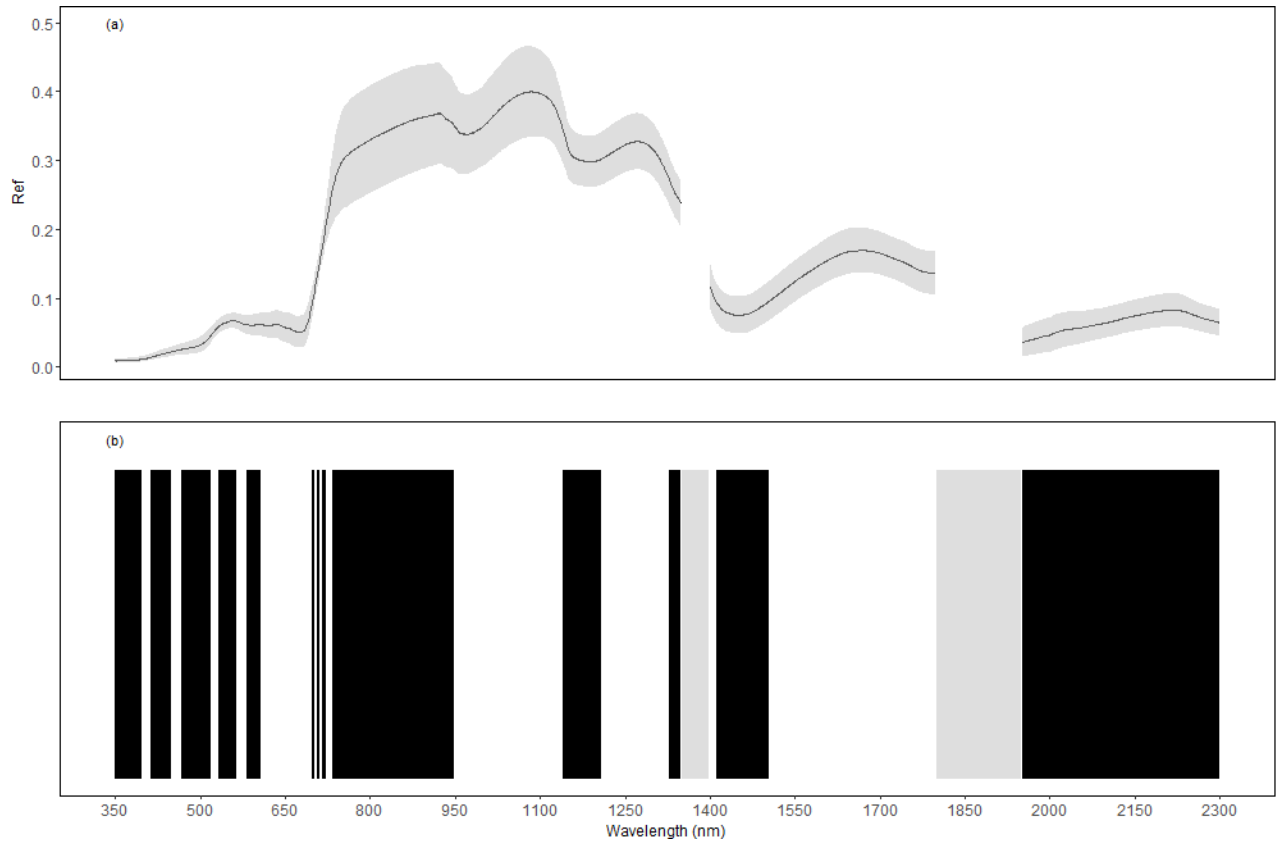

Fig.4 Average reflectance values and standard deviation (grey ribbon) observed in herbaceous plots undergoing different fertilization treatments (A). The bottom picture (B) shows the bands obtained by grouping Ref for similarity (>=90%) of contiguous hyperspectral measurements with 1nm resolution in the range 350-2300nm, bands are alternated black and white. Grey bars represent areas of the spectrum not considered for being noise.

Vegetation indices

Adopting wave bands obtained by cluster analysis (Fig. 4b) several vegetation indices were calculated (Table 2). The average values of the indices GNDVI, NDWI, PSRI and MTCI are shown in figure 5. Other indices are omitted from the figure for showing very similar trends to the ones represented (NDVI, NDVIre, WBI, CI) or not being significantly correlated with the response variable (PRI). All of them showed larger changes during the study period, particularly after April 25 (day 2), when the annual peak of growth was achieved.

The GNDVI (Fig. 5a) showed small changes among treatments and dates, with a significant drop of 20% observed from April 1 to June 3 ($P<0.000$), and significant differences in the NPK and P treatments ($P<0.001$) as compared to C but differences were not evident anymore on June 3 (day 4). The MTCI (Fig. 5b) showed a large drop particularly evident after April 25 (day 2). At April 1 (day 1), the effect of fertilization was evident in treatments NPK and P as compared to C ($P<0.001$), however along the experimental period differences among treatments diminished and by June 3 (day 4) no differences among treatments were observed. The NDWI (Fig. 5c) showed a similar temporal trend with a marked decrease from April 1 to June 3 ($P<0.001$). Also for MTCI, the NPK and P treatments showed always higher values than C ($P<0.001$), suggesting a positive impact of the higher nutrient availability on tissue water content. The PSRI had an opposite trend, showing on average threefold increase from April 1 to June 3 ($P<0.000$) and a tendency to lower values in fertilized treatments as compared to C ($P<0.001$) for NPK and P and $P<0.01$ for N).

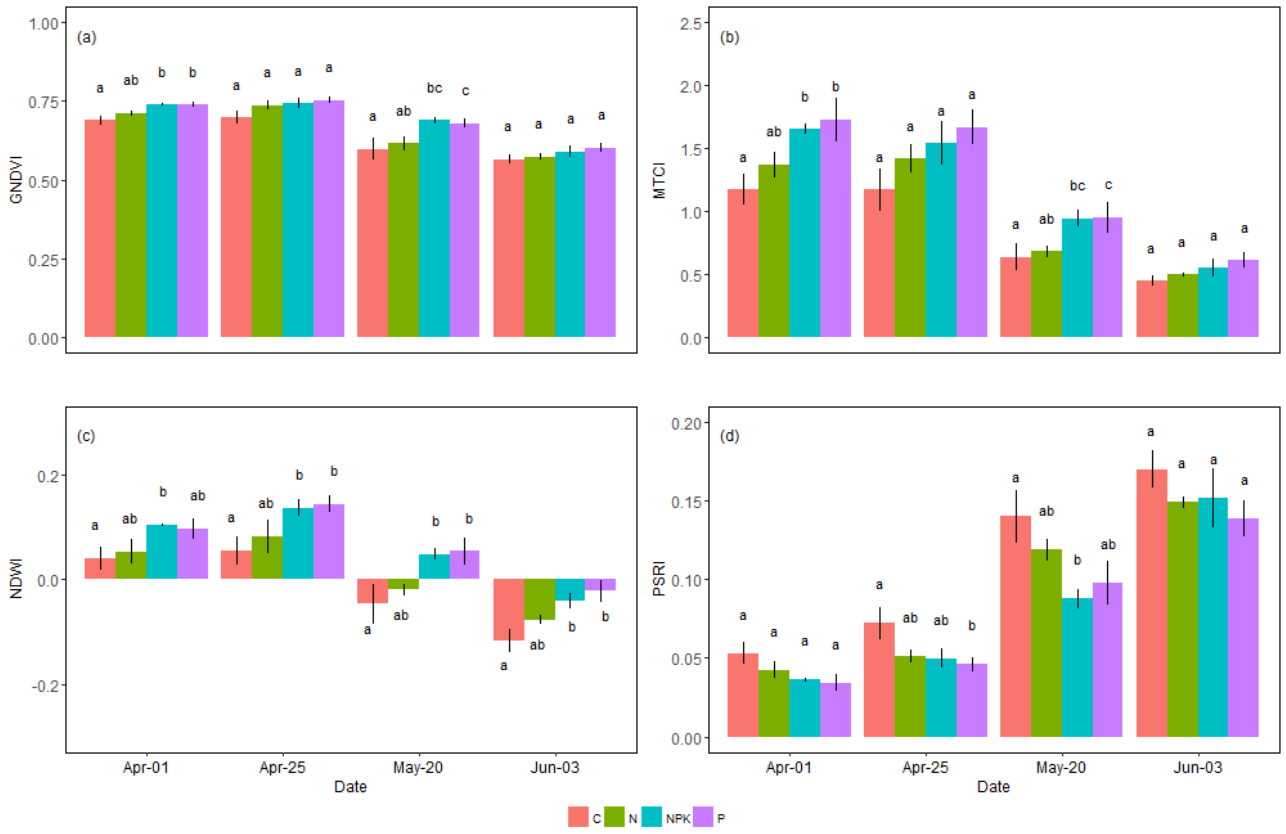

Fig. 5 Average values of several vegetation indices retrieved from reflectance measurements of herbaceous plots undergoing different fertilization treatments. Vertical bars represent standard errors.

Significant regressions were established between GPP and all the vegetation indices considered (Table 4) with the exception of PRI. The NDWI was the index that explained the higher proportion of variability of GPP, which is the result of the progressive drying out of vegetation toward the end of the growing season.

Table 4 Linear regressions established between lnGPP and vegetation indices (VI) selected for this study (see Table 2). Best regression is shown in bold.

Vegetation Index	R^2	**RMS*E***	***P***
NDVI	0.6853	0.2364	0.0000
GNDVI	0.6360	0.2543	0.0000
NDVIre	0.6872	0.2357	0.0000
CI	0.7161	0.2246	0.0000
MTCI	0.6303	0.2563	0.0000
PRI	0.0209	0.4171	0.1715
PSRI	0.6745	0.2405	0.0000
NDWI	**0.7205**	**0.2228**	**0.0000**
WBI	0.6491	0.2497	0.0000

GPP estimates by multiple linear regression models

The LEAPS procedure selected VIs or Bands as predictor variables retrieved from Hyperspectral data (Hyp) or simulating Sentinel-2 MSI (S2) and Landsat8 OLI (L8) sensors.

Models adopting only VIs as predictor variables (Hyp-VIs and S2-VIs) performed similarly with a considerable overlap of adjusted R^2 (Table S1). On the contrary, the L8-VIs model showed a lower performance (lower adjusted R^2) than Hyp-VIs and S2-VIs models (Table 5). Bootstrap results allowed us to conclude, at a confidence level of 90%, that Hyp-VIs has higher adjusted R^2 than L8-VIs.

The selection of VIs in the Hyp-VIs and S2-VIs models exhibited a similar spectral pattern. Both models included PSRI and GNDVI. On the contrary, NDVI, the most frequently adopted index as green leaf area proxy was not included in the Hyp-VIs model but only in the S2-VIs. Two of the indices included in the Hyp model are related with water balance

(WBI) and water tissue content (NDWI). The S2 model includes also MTCI, which represents chlorophyll-a and N.

Models including only bands (-B) showed similar performance to respective models employing vegetation indices (-VIs). Only in the case of L8, where just one vegetation index (NDVI) was available, bands (L8-B) led to better modelling of GPP than vegetation indices (L8-VIs)). Similar spectral patterns were also observed in the selection of bands for GPP estimate for all sensors (Hyp, S2, L8). A common pattern is the inclusion of bands in the SWIR region strongly represented in the Hyp-B ($R_{1951-2299}$, $R_{1209-1327}$, $R_{1328-1349}$), S2-B (B11) and L8-B (B6 and B7) models. The red edge region of the spectra was also largely represented in the Hyp-B ($R_{724-732,}$ $R_{706-710,}$ $R_{702-705}$, $R_{698-701}$, $R_{716-723}$) and S2-B (B5, B6 and B7) underlining the importance of this region for vegetation reflectance.

The LEAPS two-step procedure allowed to identify bands with potential to improve the VIs based models, identifying regions of the spectra generally not adopted in vegetation indices. For both Hyp and L8 the two-step model (VIs + B) increased significantly ($P<0.010$) the performance of the model, while for S2 the difference between S2-VIs and S2-VIs+B model, in spite of still significant, is less marked ($P<0.05$). The bootstrap indicated that the probability of the Hyp-VIs+B being significantly better (alpha=0.05) is 83% when compared to S2-VIs+B and 81% when compared to L8-VIs+B. On the contrary, the S2-VIs+B and the L8-Vis+B models exhibit roughly the same explanatory power. In all the VIs+B models, bands in the SWIR region were included. The second region of the spectra more represented in the Hyp-B and Hyp-VIs+B model was the red-edge.

Discussion

Best VIs for GPP estimation

The LEAPS procedure selected several indices as significant predictor variables for GPP in the Hyp-VIs and S2-VIs model (Table. S1). The vegetation indices selected in the Hyp-VIs and S2-VIs models are known to represent different properties of vegetation, specifically: the green fraction of the leaf area (GNDVI and NDVI), the chlorophyll-a and N concentration (MTCI), the ratio carotenoids/chlorophyll (PSRI) and the tissue water content (NDWI,WBI). Each of these traits has a major role in GPP.

Among the vegetation indices selected in the multiple linear models, both the Hyp-VIs and the S2-VIs included the PSRI (Merzlyak et al., 1999) which is generally applied to detect the occurrence of vegetation senescence. PSRI is able to capture changes in the carotenoids/chlorophyll ratio which occur during vegetation senescence since chlorophyll declines more rapidly than carotenoids (Merzlyak et al., 1999). In this study, PSRI increased in all treatments after April 25, where the maximum peak of growth (Fig. 2,b) was achieved and close to the onset of canopy drying out. Another index known to be related with the carotenoids/chlorophyll ratio, the PRI (Filella et al., 2009), showed no correlation with GPP in our study. These results are in contrast with previous studies (Perez-Priego et al., 2015). However, a low performance of PRI in representing the carotenoids/chlorophyll ratio has been already observed in semiarid grasslands (Vicca et al., 2016). In crops, a good agreement between PRI and pigment pools was observed at leaf (Gitelson et al., 2017a) but not at stand level (Gitelson et al., 2017b). Differences in the last two studies were ascribed to changes in canopy structure (e.g., changes in leaf inclination angle) over the growing season.

The Hyp model also put in evidence the importance of changes in canopy water content, as both the NDWI (Gao, 1996) and the WBI (Penuelas et al., 1997) were included in the model. Changes observed in NDWI along the experiment and the good correlation observed between NDWI or WBI and GPP is an evidence of the importance of the onset of drought for grassland vegetation as senescence marks the end of the growing season in Mediterranean grasslands (Balzarolo et al., 2015; Vescovo et al., 2012). In a previous study, Vicca (et al., 2016) found that NDWI was able to estimate GPP in semiarid grasslands better than other indices, allowing to distinguish the effect of drought.

Other indices, sensitive to changes in chlorophyll-a concentration, MTCI (Dash and Curran, 2004) and GNDVI (Gitelson and Merzlyak, 1998) were also included in the model. The fertilization treatment resulted in an increase in MTCI during the first stage of the experiment in the NPK and P treatments, followed by a decrease observed in all treatments as the season progressed toward the end of the annual growth cycle. A similar trend was observed in a study by (Perez-Priego et al., 2015) in which Mediterranean grasslands were subjected to fertilization with N or NP. The primary role of chlorophyll in photosynthesis is well known and justifies the positive relationship observed between GPP and MTCI. However, in the present study, no differences were observed in GPP among fertilized and non-fertilized treatments suggesting that the expected increase in

photosynthesis due to the increase in chlorophyll and nitrogen was constrained by other environmental and physiological factors.

Notably NDVI, the most frequently applied index in GPP estimates by LUE models (Yuan et al., 2014) was not selected in the Hyp-VIs model and showed a poorer coefficient of determination than other indices, (e.g. NDWI), NDVI is expected to mirror changes in green leaf area, being generally linearly related with fPAR (Myneni and Williams, 1994). However, previous studies reported a saturation of NDVI and consequent lack of linearity in the NDVI-fPAR regression in high productive vegetation communities (Gianelle et al., 2009; Vescovo et al., 2012) such as grasslands and sometimes other indices showed a better performance. For example, in grasslands subjected to water and nutrient stress, the NDVI green index (GNDVI), which adopts a green band instead of the red band of NDVI and hence is more sensitive to chlorophyll-a concentration (Gitelson et al., 1996), showed a better performance than NDVI as fPAR proxy (Cristiano et al., 2010; Gianelle et al., 2009). Also in this study, the GNDVI explained a larger proportion of GPP variance than NDVI in the Hyp-VIs and S2-VIs models being selected in both and before NDVI in the S2-VIs model.

The indices selected by the LEAPS (i.e. NDVI, GNDVI, NDWI, MTCI, PSRI and WBI) also showed high significant relationship with GPP (tab. 4) in simple regressions explaining 63% to 72% of the variability observed. The functional convergence (Ollinger, 2011) of different traits participating in the photosynthetic process may have hampered results observed in the regression for each single vegetation index, showing a high degree of correlation for most of them (Table 4). However, the selection of several VIs, representatives of different structural and functional traits in the multiple linear models and the lower performance observed in the L8 model, including solely the NDVI index, clearly indicate the importance of considering the contribution of different traits with different temporal dynamics to capture GPP temporal changes in models integrating vegetation indices.

Are spectral bands better GPP estimators than VIs?

Our results suggest that better GPP estimates can be obtained by adopting bands (Hyp-B, S2-B, L8-B, Table 5) instead of vegetation indices (Hyp-VIs, S2-VIs, L8-Vis, Table 5).

Although normalized VIs are important in establishing strong relationships between biophysical and optical properties of vegetation, our results showed that the selection of the proper band is more important than the mathematical formulation of the indices for the explanatory power of spectral retrievals as predictor variables. Previous studies comparing the explanatory power of VIs and bands in grasslands showed similar results (Balzarolo et al., 2015; Matthes et al., 2015).

Our results also evidenced the importance of the SWIR region of the spectra, as bands in this region were selected in all one- and two-step models, which is rarely adopted in vegetation indices with few exceptions. The SWIR region is known to correlate with canopy water content (Casas et al., 2014).

Studies investigating the potential of spectral bands to estimate canopy chlorophyll content and green *f*APAR, found that the SWIR region was strongly positively correlated with them in grasslands (Sakowska et al., 2016) and also GPP in a semi-arid savanna (Tagesson et al., 2015).

Bands in the red-edge region were also largely represented in the Hyp-B, S2-B and in the Hyp-VIs+B models. The red-edge corresponds to the steep increase in reflectance at the boundary between the red region where chlorophyll is absorbed and the leaf scattering at the NIR region. Red-edge bands were successfully employed for estimating chlorophyll content in maize (Zhang and Zhou, 2017), LAI in crops (Kira et al., 2017). For these reasons they were integrated into numerous VIs, such as MTCI and PSRI, also applied in this study, which explain the reason for the lack of red-edge bands in the second step of the S2 model (S2-VIs+B) while strongly represented in the S2-B.

Satellite sensors as estimators of GPP

Differences in the selection of the vegetation indices among sensors had apparently no effect on the performance of the S2-VIs and Hyp-VIs models (Table 5), while the limited number of available vegetation indices for the L8 resulted in a lower performance of the model.

Our results show an equal potential of S2 and L8 sensor for assessing GPP.

GPP estimates obtained simulating S2 and L8 sensors showed a similar performance in the –B and –VIs+B models, while when only VIs were adopted the S2 model had clearly a better performance than L8. These results suggest a need for testing new vegetation indices adopting L8 bands.

In agreement with our results, other studies comparing linear additive models showed similar ability for estimating canopy cover and LAI adopting the S2 or L8 sensors (Korhonen et al., 2017).

An important difference between the S2 and L8 availability of wavebands is the lack of reflectance values in the red-edge region in the L8, which limited the possibility of computing VIs, such as MTCI and PSRI (Korhonen et al., 2017). However, the limitation imposed by the lack of bands in the red-edge region, had apparently more importance for the -VIs model, while differences in the performance of the model between S2 and L8 decreased for –B and VIs+B models.

In this study, S2 and L8 data comparison was based only on the simulation of the respective bands not taking into consideration other factors possibly affecting sensors spectral response such as sun-sensor viewing geometry (Tagesson et al., 2015). Nonetheless, in a recent study (Korhonen et al., 2017) the comparisons of satellite data from the two platforms showed no differences between S2 and L8 reflectance values in the NIR, SWIR1 and SWIR 2 bands. In other regions of the spectra, such as the green and blue bands reflectance values were considerably smaller in the S2 than in the L8 but still proportional, suggesting that comparisons between S2 and L8 simulated bands can largely be representative of the actual differences obtained by the two remote platforms.

At the same time, our results confirm the importance of performing hyperspectral measurements. Indeed, in this study, the Hyp-VIs+B model showed to be superior to the corresponding S2 and L8 model with over than 80% of probabilities. The high detailed resolution and the wide range if wave bands makes hyperspectral sensors as unique in identifying regions of the spectra of high interest for representing different properties of vegetation (Porcar-Castell et al., 2015)

Conclusions

In agreement with previous studies(Perez-Priego et al., 2015; Rossini et al., 2012; Vicca et al., 2016), our results clearly indicate the need to integrate into GPP models spectral information representing both structural and functional traits of vegetation along the whole grasslands life cycle. Specifically, water content (NDWI), chlorophyll (MTCI, GNDVI) and the ratio of chlorophyll to carotenoids (PSRI) were indicated as best predictor variables for GPP estimates. Altogether these vegetation indices describe the loss of photosynthetic pigments and efficiency and dry out of vegetation occurring and when considered together improved considerably GPP estimates in comparison with models adopting only NDVI.

Our study also confirms the importance of hyperspectral in-situ measurements for exploratory analysis of the relationship between biophysical and optical properties of vegetation providing a wide spectral range and high resolution of spectral retrievals.

The hyperspectral reflectance values, together with the two-step procedure adopted for the selection of predictor variables allowed also to identify critical region of the spectra, not included in the initial selection of vegetation indices but that revealed their usefulness in estimating GPP. For example, the LEAPS two-step procedure evidenced which bands could improve significantly a model including only vegetation indices, identifying the red edge and SWIR regions of the spectra as of major importance for improving GPP estimates. This information can be critical in the development of new spectral indices and sensors.

Our results also evidenced the potential of S2 and L8 sensor in assessing GPP, since models obtained by simulating bands from the two sensors showed similar performance. The possibility of using remote sensing information for monitoring and modelling vegetation at a suitable spatial resolution, such as in S2 and L8 sensor, allows attempted vegetation monitoring and modelling in a cost-effective way, in support of sustainable agriculture management practice.

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Supporting information

Table 5. Best selection of linear models for GPP estimate according to the general equation: $lnGPPP \sim \sum_{j,1}^{n} vj$, where v are vegetation indices (VIs) or optical bands (B). Bands and vegetation indices are obtained from Hyperspectral measurements grouped in clusters with 90% similarity (Hyp), or resampled for simulating Sentinel 2/MSI (S2) and Landsat8/OLI (L8) sensors. Vegetation indices formulation is shown in Table 2. The order of the variables (most important first) reflects their importance in the model. The Quantiles 25% and 75% of the *adj* R^2 are obtained from a bootstrap with 10000 iterations. The two-steps models add a selection of bands to the variables (VIs) selected at step one. A low p-value indicates that the model including VIs and bands (step 2) is significantly better than the model just with VIs (step 1).

Model	Step one	*Adj* R^2	*Adj* R^2 *Q-25%*	*Adj* R^2 *Q-75%*	Step two	*Adj* R^2	*Adj* R^2 *Q 25%*	*Adj* R^2 *Q75%*	*p*
Hyp-VIs	NDWI; PSRI; WBI; GNDVI	0.7659	0.7431	0.8047					
S2-VIs	MTCI; PSRI; GNDVI; NDVI	0.7426	0.7225	0.7822					
L8-VIs	NDVI	0.6792	0.6405	0.7194					
Hyp-B	$R_{1951\text{-}2299}$; $R_{724\text{-}732;}$ $R_{1328\text{-}1349}$; $R_{706\text{-}710}$; $R_{449\text{-}466}$; $R_{566\text{-}582}$; $R_{519\text{-}532;}$ $R_{350\text{-}397}$; $R_{398\text{-}411;}$ $R_{1209\text{-}1327;}$ $R_{702\text{-}705}$; $R_{698\text{-}701}$; $R_{716\text{-}723}$;	0.7884	0.7906	0.8392					
S2-B	B7; B11; B5; B2; B8; B6	0.7412	0.7222	0.7848					
L8-B	B7; B5; B6; B4; B3; B1	0.7557	0.7367	0.7974					
Hyp-VIs+B	NDWI; PSRI; WBI; GNDVI				$R_{698\text{-}701}$; $R_{412\text{-}448}$; $R_{716\text{-}723}$; $R_{467\text{-}518}$; $R_{706\text{-}710}$; $R_{449\text{-}466}$; $R_{350\text{-}397}$; $R_{1209\text{-}1327}$; $R_{1412\text{-}1505}$; $R_{1951\text{-}2299}$; $R_{702\text{-}705}$; $R_{724\text{-}732}$; $R_{1328\text{-}1349}$	0.7986	0.8083	0.8550	0.0260
S2-VIs+B	MTCI; PSRI; GNDVI; NDVI				B11; B3; B12	0.7684	0.7542	0.8104	0.0081
L8-VIs+B	NDVI				B6; B3; B7	0.7686	0.7472	0.8047	0.0000